Experimental Evolution of a Freshwater Isolate with Inducible Aggregate Formation – Genome Streamlining and Variability of Phenotypic Plasticity

Dissertation

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We live now in the "Age of Bacteria." Our planet has always been in the "Age of Bacteria," ever since the first fossils—bacteria, of course—were entombed in rocks more than 3 billion years ago.

On any possible, reasonable or fair criterion, bacteria are—and always have been—the dominant forms of life on Earth.

Stephen J. Gould

Summary

Predator-prey relationships between bacteria and their predators might be one of the oldest and, from an ecological and evolutionary point of view, most influential interactions in nature. Predation impacts microbial population densities and structure and is a driver of biogeochemical nutrient cycling. Bacteria evolved innumerable ways to avoid being ingested by a predator. These strategies involve morphology changes, cooperative behaviour or the release of toxins, to mention only a few. Despite its impact on the ecosystem, the long-term effects and evolutionary trajectories of predation by protists on bacteria are still poorly understood.

I used an experimental evolution approach to get a better understanding of the evolutionary path of a bacterial prey in a model predator-prey system. I found that constant presence or absence of a flagellate predator under substrate limiting conditions shifted the phenotypes of evolved isolates of *Sphingobium* sp. Z007 either to enhanced aggregate formation or to suppression of aggregation. Furthermore, whole genome sequencing revealed that the combination of substrate/nutrient reduction and high mortality had a more pronounced impact on genome evolution than oligotrophy only. *Sphingobium* sp. Z007 evolved under oligotrophic conditions in the presence of predators reduced its genomic content, which resulted in higher growth efficiency despite enhanced aggregate formation. I also found that the phenotypic plasticity that renders sensitivity to predators is a fragile trait that is rapidly lost when the trigger for the defence morphotype is continuously present or absent. We could show that only a sporadic confrontation with predators maintains the predator sensing ability and consequently the phenotypic plasticity of the population. Finally, the co-cultivation of bacteria and flagellates on rich media over a longer time period had a beneficial effect on the population density that could be reached by evolved isolates.

I thus provide experimental evidence that the combination of mortality and nutrient limitation does not necessarily lead to an either/or adaptation for a growth or defence specialist but rather to the circumvention of the trade-off by exploiting a strategy to simultaneously adapt to both types of selection. My findings suggest that one such strategy is to jettison large stretches of DNA from an integrated plasmid, at the cost of losing metabolic functions that are not relevant in the selective scenario. Furthermore, I experimentally illustrated that the dynamics of environmental fluctuations may influence the phenotypic plasticity of a population. Taken together, these findings help to gain a better understanding how bacteria cope with opposing selection constraints and environmental heterogeneity at an ecologically relevant time scale of microevolutionary adaptation.

Zusammenfassung

Mikrobielle Räuber-Beute Beziehungen sind wahrscheinlich eine der ältesten und von einem ökologischen und evolutionären Standpunkt aus bedeutendsten natürlichen Interaktionen. Frassfeinde beeinflussen die bakterielle Populationsdichte, -struktur und sind ein Motor für biogeochemische Nährstoffkreisläufe. Um den Fressfeinden entgegenzuwirken, evolvierten Bakterien unzählige Strategien, um sich gegen Räuber zu verteidigen. Dazu zählen Änderungen der Morphologie, kooperatives Verhalten oder die Bildung von Toxinen. Trotz ihrer Wichtigkeit für das Ökosystem sind die evolutionären Auswirkungen von Räuber-Beute Beziehungen auf Bakterien noch nicht sehr gut untersucht.

Während dieser Doktorarbeit untersuchte ich unterschiedliche Interaktionen zwischen Mikroorganismen und ihrer Umwelt in einem öko-evolutionären Zusammenhang und nutzte dafür den Ansatz der experimentellen Evolution eines Räuber-Beute Systems. Ich konnte zeigen, dass konstante An- oder Abwesenheit eines Räubers unter nährstofflimitierenden Bedingungen die Zell-Aggregation in so evolvierten Stämmen des Bakteriums *Sphingobium* sp. Z007 entweder erhöht (konstanter Frassdruck) oder unterdrückt. Genomsequenzierung des Ursprungsstamm und der evolvierten Stämme zeigte, dass eine Kombination von hoher Mortalität und Nährstofflimitierung einen grösseren Einfluss auf das Genom hatte als Oligotrophie allein. *Sphingobium* sp. Z007, evolviert unter nährstoffarmen Bedingungen und hohem Räuberdruck, konnte durch Genomreduktion trotz erhöhter Aggregation eine grössere Wachstumseffizienz erreichen. Des Weiteren stellte ich fest, dass die phänotypische Plastizität, welche die Aggregatbildung bei Anwesenheit von Räubern verstärkt, ein fragiler Phänotyp ist, welcher schnell verloren geht, wenn der Auslöser für diese Plastizität (der Räuber) durchgehend an- oder abwesend ist. Ich konnte zeigen, dass nur eine sporadische Konfrontation mit dem Räuber die Fähigkeit auf den Räuber zu reagieren aufrechterhält und damit verbunden auch die phänotypische Plastizität. Die Ko-kultivierung von Bakterien mit dem Räuber über einen längeren Zeitraum hatte immer einen positiven Effekt auf die Gesamtzelldichten der evolvierten Stämme.

Ich konnte damit experimentell zeigen, dass die Kombination von Mortalität und Nährstofflimitierung nicht ultimativ zu einem Kompromiss zwischen verschiedenen Anpassungen (‚trade-off') führen muss, sondern ein derartiger evolutiver Konflikt auch durch eine gleichzeitige Optimierung für beiden Faktoren umgangen werden kann. Eine Möglichkeit, um sich gleichzeitig an zwei gegenläufige Selektionszwänge anzupassen, ist die Reduktion der Genomgrösse. Dies mag zwar mit dem Verlust von Eigenschaften einhergehen, diese können aber in gewissen Selektionsszenarien irrelevant sein. Die Ergebnisse dieser Arbeit helfen uns besser zu verstehen, wie Bakterien mit diametral verlaufenden Selektionszwängen in einer heterogenen Umwelt umgehen, und zwar in einem Zeitrahmen, der sowohl für ökologische Interaktionen als auch für mikroevolutionäre Adaption relevant ist.

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1 Introduction

1.1 Predator-prey interactions

Predation is one of the important ecological factors that influence microbial community structure and composition in aquatic systems (Gasol et al. 2002). One of the oldest and possibly most fundamental predator-prey relationship is realized between bacteria and their consumers (Jürgens and Güde 1994). Three main groups of bacterivorous predators (including parasites) can be distinguished in aquatic systems: predatory bacteria such as bdellovibrios or myxobacteria, phages, and protists (Shilo and Bruff 1965, Wommack and Colwell 2000, Pernthaler 2005, Berleman and Kirby 2009). To some extent, larger eukaryotes such as rotifers and cladocerans may also consume bacteria, e.g. filamentous forms or larger bacterial assemblages (Vaqué and Pace 1992, Jürgens et al. 1999, Jürgens and Jeppesen 2000).

Phagotrophy by protists and lysis by phages are considered the main mortality factors for planktonic bacteria (Pernthaler 2005). These top-down factors affect total bacterial abundances and biomasses (Pernthaler et al. 1996), and the taxonomic (Simek et al. 1999) and functional structure of bacterial communities (Rønn et al. 2002). Bacteria are at the base of the heterotrophic food chain, and they are also important drivers of nutrient recycling. This complex interplay between microbial predators and their prey has first been described by the microbial loop concept (Fig.1), wherein bacteria transform the dissolved organic matter (carbon and nutrients) into particulate form and thus make it available for higher trophic levels (Azam et al. 1983). More recently, lysis by bacteriophages (the ‘viral shunt’) has also been incorporated into this model (Wilhelm and Suttle 1999).

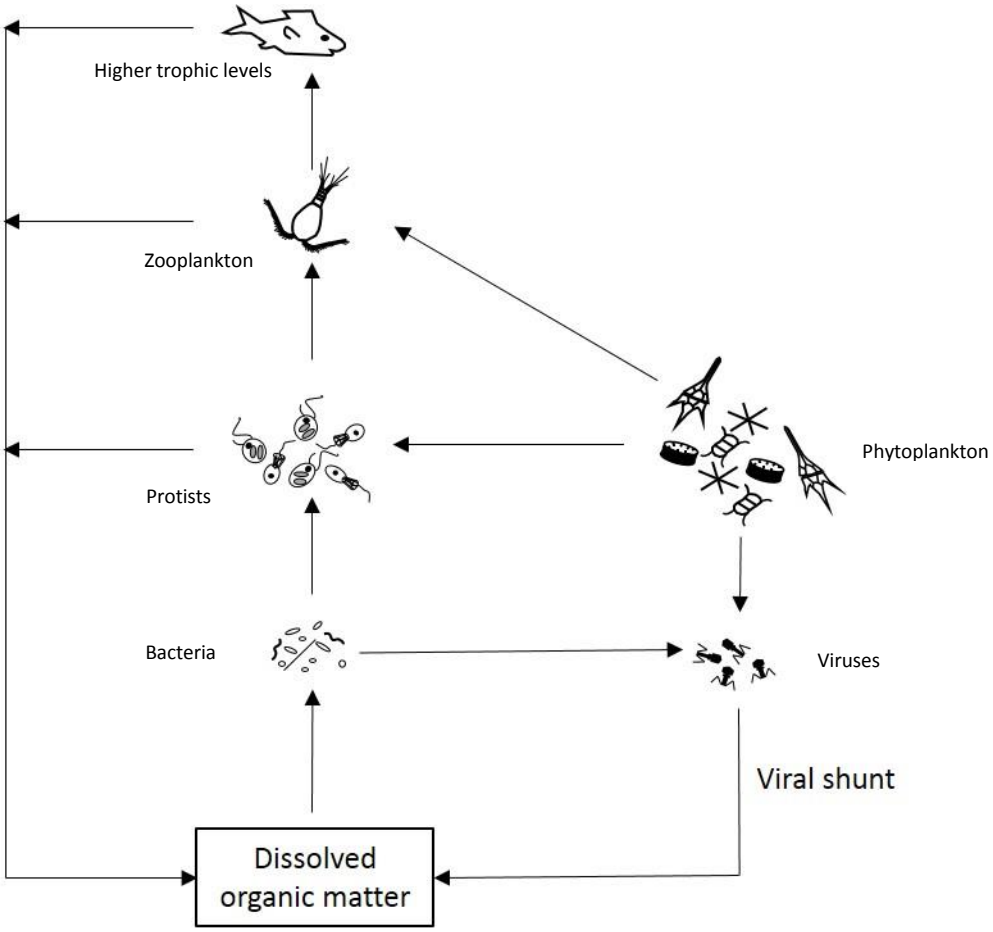

Fig. 1: Schematic drawing of the microbial loop including the viral shunt in the aquatic food chain.

Being able to avoid predation improves bacterial survival in top-down controlled communities. This has resulted in the evolution of numerous different strategies to resist grazing (Matz and Kjelleberg 2005). The foraging process is composed of the individual steps of finding, recognition and ingestion of the prey (Jeschke et al. 2002). Bacteria have evolved specific adaptations that are designed to impede specific stages of the predation process. For example, prey can avoid being localized by inhabiting refuges such as biofilms that are inaccessible to predators (Postma et al. 1990, Jousset et al. 2006). In addition, bacteria mask their traces by interfering with the chemotaxis system of the predators, or they produce deterrents and toxins to increase its search time (Jousset et al. 2006, Pradel et al. 2007). Once encountered by the predator, a potential prey has to fit within its food spectrum. In bacteria-flagellate interactions, prey is mainly selected according to its size (Jürgens and Šimek 2000). As a consequence prey morphotypes that are beyond the size spectrum of predators have a higher chance of survival (Pernthaler 2005). Heterotrophic nanoflagellates, the main mortality

source for planktonic bacteria, are typically not able to consume prey that is larger than approximately 4-5 µm cell length (Matz et al. 2002). Therefore, cell types such as filaments (Hahn and Höfle 1999, Corno and Jürgens 2006), or growth forms such as suspended microcolonies (Hahn et al. 2000), aggregates (Blom et al. 2010a) or biofilms (Matz et al. 2005) can be considered as phenotypes that also evolved to decrease mortality by predation (Fig. 2).

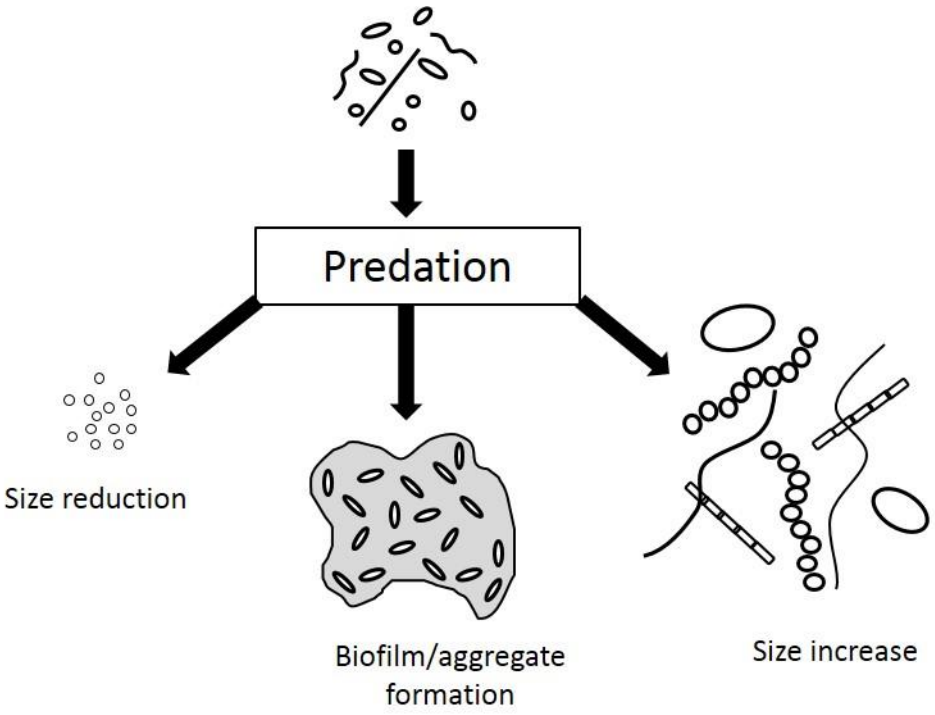

Fig. 2: Morphological alterations of bacteria in response to predation

Some bacteria have an innate predator protection due to their permanently small or large cell size (Pernthaler et al. 2004, Salcher et al. 2011). However, the expression of predator-protected phenotypes is considered costly (Callahan et al. 2008) due to resource allocation for production of secondary metabolites or the impeded substrate uptake upon morphological shifts like aggregation (Jousset 2012). Therefore, it is an advantageous strategy of bacteria to form predation protected phenotypes only in presence of a mortal threat. Previous studies suggested that chemical cues released by the predator can be sensed by some bacteria and lead to drastic phenotypic shifts (Corno and Jürgens 2006, Blom et al. 2010b). This part will be further explained in context of our model organism in the following chapter.

The above mentioned innate costs of predator defence and the resulting trade-off between resistance and performance have a direct impact on the competition

between predation-sensitive and protected strains, which in turn are dependent on predator density and resource availability (Muyzer and Ramsing 1995). The interplay and influence of these bottom-up and top-down factors on natural communities are still poorly understood (Pernthaler 2005). However, it is known that predators have a strong impact on prey diversity. Sir Arthur George Tansley, an English botanist of the early 20th century, made the observation that ungrazed vegetation was overgrown by fast growing plants when he excluded rabbits from a patch of grassland, with the consequence of reduction in diversity. By contrast heavy grazed grassland showed a much higher species diversity (Tansley and Adamson 1925). In the aquatic habitat flagellate predators with their preference for unprotected organisms and size selective foraging behaviour remove the most abundant and rapidly growing bacterial strains and help to maintain bacteria with less competitive strength in the system (Beardsley et al. 2003). Thingstad concluded this observation in the “killing the winner” theory, which suggests that the most abundant bacterial prey is under negative density dependent selection, which has a positive influence on the total bacterial diversity (Thingstad 2000).

The strong impact of predation may shape bacterial evolution by selecting for resistant phenotypes, which could explain the high diversity found in bacterial communities (Lubin et al. 2001). A driver of this predation triggered evolutionary process might be enhanced mutation rates (Pal et al. 2007) or horizontal gene transfer (Vos 2009). For example pyrrolnitrin, a secondary metabolite of, e.g. *Pseudomonas* that is toxic for protists, was shown to be acquired by many different bacterial strains via horizontal gene transfer (Costa et al. 2009, Jousset et al. 2010). It seems also possible that predators can mediate resistance against other predators as it was demonstrated that bacteria carrying the Coliphage T4 showed enhanced resistance against infection by other bacteriophages (Labrie et al. 2010).

Although the expression of resistant phenotypes might be associated with costs on different levels, there are other potent mechanisms, which diminish this ostensible disadvantage. Some defence mechanisms can be coupled to other growth-related functions and therefore have a positive pleiotropic effect. Defence traits such as the increased motility or enhanced biofilm formation are also involved in other cellular processes. For example, biosurfactants that are important for biofilm formation and spreading of bacterial populations, have been shown to make hydrophobic compounds

(such as polycyclic aromatic hydrocarbons) better available for bacteria (Tecon and van der Meer 2010). Another aspect is to entirely re-consider the commonly assumed trade-off between being an “uptake” or “defence” specialist: Thingstad hypothesized that these two phenotypes might not necessarily have to exclude each other (Thingstad et al. 2005). He suggested a model, where bacteria are using an available non-limiting substrate (glucose) to increase cell volume, thereby bypassing the trade-off of nutrient limitation and predation.

Predator-prey interactions play a central role in nutrient cycling and have strong evolutionary consequences. Many of the bacterial morphologies found in nature might be a result of predation. Furthermore, predator-prey interactions keep the ecosystem going by lifting energy from the lowest to the top trophic level. Therefore, in analogy with the famous statement by Dobzhansky one might say that: “only in the light of predation many things in microbiology start to make sense”.

1.2 Model prey organism: *Sphingobium* sp. Z007

Our model organism *Sphingobium* sp. Z007 is affiliated with *Sphingobium,* a genus from the family *Sphingomonadaceae* in the subphylum Alphaproteobacteria. The family of *Sphingomonadaceae* was originally proposed based on the 16s rRNA gene sequence phylogeny and due to the presence of sphingoglycolipid as their characteristic membrane lipid (Kosako et al. 2000). Sphingoglycolipids are found in eu- and prokaryotes and have important physiological functions in substituting lipopolysaccharides in the bacterial cell walls (Kawahara et al. 1991). Another phenotypic characteristic of this family is the bright yellow pigmentation of colonies due to carotenoids (Yabuuchi and Kosako 2015). These pigments may play an important role in the fluidity of the membrane and reduction of oxidative stress caused by the degradation of heterocyclic compounds, another common feature of the *Sphingomonadaceae* (Liu et al. 2012). Genome sizes of this family range from 2.1 to 5.9 Mbp with an average G+C content between 41 and 68 % (Glaeser and Kämpfer 2014). Genome organisation is usually one or two chromosomes and one to five plasmids. Degradation pathways for complex substrates are often found on plasmids or partially integrated on chromosomes (Basta et al. 2005). Furthermore, there are indications that horizontal gene transfer is common in this family (Aylward et al. 2013). Sphingomonads have been isolated from a broad range of habitats, including soils

contaminated with aromatic compounds (Willison 2004), freshwater and marine habitats (Cavicchioli et al. 2003, Jogler et al. 2011), phyllosphere (Vorholt 2012) and rhizosphere (Chapelle et al. 2016). The genera of *Sphingomonas, Novosphingobium and Sphingobium*, isolated from drinking water supply, have high tolerance to a wide range of antibiotics, especially to those of the beta-lactam or peptide type (Vaz-Moreira et al. 2011). It was therefore hypothesized that Sphingomonads are a source for clinical relevant antibiotic resistance. *Sphingomonadaceae* are also interesting for two fields of application: Due to their special degradation capabilities they can be potentially used in bioremediation of contaminated aquifers or soils (Tiirola et al. 2002, Liu et al. 2015). Secondly, their production of exopolymeric substances might have a variety of possible applications in industry (Sutherland 2001).

Sphingobium sp. Z007 was originally isolated from the oligomesotrophic Lake Zurich, by incubating substrate-amended surface lake water with the flagellate predator *Poterioochromonas* sp. strain DS and subsequently plating. It is a rod shaped, yellow-pigmented and mobile bacterium, which has a planktonic and an aggregated / biofilm lifestyle. *Sphingobium* sp. Z007 forms freely floating cell aggregates or microcolonies that by far exceed the ingestible size of a model predator (see below) (Blom et al. 2010a). Moreover, supernatants of bacteria-predator co-cultures have an aggregation inducing effect (Blom et al. 2010b). This suggests that a chemical compound triggers aggregation and serves as a kairomone to warn bacteria of the imminent threat. Since *Sphingobium* sp. Z007 can be readily maintained at -80°C and grows rapidly on complex media it is a good model organism for experimental studies on predator-prey interactions.

Many examples of predator-induced behavioural or morphological changes are known, such as the helmet formation in *Daphnia* (Laforsch and Tollrian 2004) or filamentation in some bacterial species, e.g. *Flectobacillus* (Corno and Jürgens 2006). Although the inducibility of these and other morphotypes in a variety of (micro)organisms has been known for some time, the elucidation of responsible compounds has not yet been successful. A recent study identified a specific compound of the bacterial cell wall, a sulfonolipid, that triggers rosette formation in a flagellate in presence of its bacterial prey (Alegado et al. 2012). It was hypothesized that this multicellular structure increases the predation efficiency of the flagellate (Beemelmanns et al. 2014).

1.3 Model predator *Poterioochromonas* sp. strain DS

Poterioochromonas sp. DS is a mixotrophic nanoflagellate belonging to the *Chrysophyceae*. The strain was isolated from Lake Constance and originally classified as *Ochromonas* sp.; this was corrected to *Poterioochromonas* after the sequencing of its 18s rRNA gene (Boenigk and Arndt 2002). The feeding on bacteria is the most important energy source of this mixotroph, whereas only 3% of the total carbon demand is met by photosynthesis (Boenigk et al. 2006). *Poterioochromonas* sp. DS is an interception feeder (Fig. 3), which captures bacteria by means of its flagella. Prey items are then enclosed by a membrane and digested in a vacuole (Boenigk and Arndt 2002). Assuming minimal densities of bacteria of around 10^6 ml^{-1} flagellates can capture between 5-10 bacterial cells per hour (Boenigk and Arndt 2002).

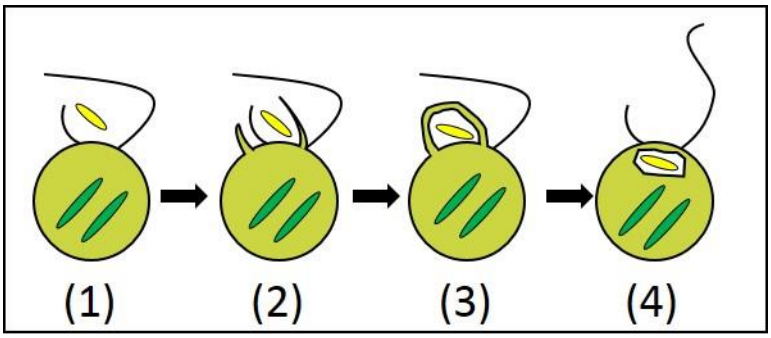

Fig. 3: Feeding phases of *Poterioochromonas* sp. DS. (1) Encounter/recognition, (2) handling and adjustment, (3) formation of food vacuole and (4) digestion of the prey. Modified from (Boenigk and Arndt 2002).

1.4 Experimental evolution

In his paradigm-shifting treatise *On the Origin of Species by Means of Natural Selection* Darwin stressed that evolution is an ongoing process. However, for many decades evolutionary biologists had to rely on approaches where living organisms were compared with the fossil record (e.g. Patterson 1981) or by means of molecular phylogenies (e.g. Morris 1998). The disadvantage of these methods is that one can only reconstruct past events of evolution but never observe an ongoing evolutionary process. However, already soon after the publication of Darwin's evolutionary theory scientists came up with ideas for experiments to observe evolution in action. One of

the first who showed that it is possible to observe the process of evolution was a Methodist clergy man named William Dallinger. The reverent bred protists under an increasing temperature regime where they adapted to temperatures that were twice as high as the initial incubation temperature (Dallinger 1878). Henry de Varigny approached the topic from a more theoretical point of view: in his book *Experimental Evolution* he suggested long-term experiments which would cover the lifespan of the conducting scientist (De Varigny 1892). As of late, this concept has been realized to some extent in the career and research of Richard Lenski (Fox and Lenski 2015). After the turn of the 20th century *Drosophila* had become the organism of choice for experimental population genetics, in order to investigate the influence of selection and genetic drift in laboratory populations (Morgan 1910). Bacteria were still not favoured at this time because of two reasons. For one, there was a lack of suitable technologies to efficiently investigate bacterial genetics. As a consequence scientist could observe the rapid changes bacteria underwent under certain conditions, but the underlying mechanisms seemed to be a confusing mix of Lamarckian and Darwinian evolution (Lewis 1934). The rise of bacteria in evolutionary science was probably due to the ground-breaking experiment of Salvador Luria and Max Delbrück, who showed that mutations occur spontaneously in bacteria and selection retains the ones that are useful (Luria and Delbrück 1943). In the 1940s continuous cultures started to be used to study bacteria, but the motivation of these experiments was more of fundamental physiology than evolutionary, e.g. to investigate growth rates under limiting conditions (Bryson and Szybalski 1952). Nevertheless, this period can be seen as the dawning of experimental evolution of microbes. According to the definition of Kawecki et al. (Kawecki et al. 2012) experimental evolution is "*the study of evolutionary changes occurring in experimental populations as a consequence of conditions imposed by the experimenter*." Consequently, evolutionary changes that do not result from planned experiments, such as the loss of traits upon extended cultivation on particular media, are not considered (Kawecki et al. 2012).

These early experiments on microbes have provided much insight into basic molecular mechanisms, such as the importance of gene regulation, presence of mobile elements and large scale genomic changes, which had a broader influence on the general understanding in biology (Adams and Rosenzweig 2014). More recently, the discovery of high bacterial diversity by Norman Pace, Steve Giovannoni, David Stahl and others on the one hand (Stahl et al. 1985, Giovannoni et al. 1990, Pace 1997),

and the rapid evolution of antibiotic resistance on the other (Palumbi 2001), has increasingly drawn the attention of evolutionary biologists to microbes as model organisms (e.g. Perron et al. 2006). There are three major advantages to the use bacteria in evolutionary experiments 1) they have short generation times, 2) their growth environment can be strictly controlled and 3) they can be cryopreserved, so that ancestral lineages are available for comparison (Barrick and Lenski 2013). In the 1980s researchers started to investigate the evolution of predator-prey interactions (Lenski and Levin 1985) and the influence of long-term cultivation under nutrient restricted conditions (Lenski et al. 2003). Since then, many evolution experiments using a wide variety of model microorganisms have been conducted (Rainey and Travisano 1998, Rogers and Greig 2009, Chou et al. 2011). The improvement of sequencing technologies has been another of the reason why this approach has recently become so popular (Adams and Rosenzweig 2014).

Experimental evolution has yielded valuable insight into how (micro)organisms adapt to specific environments (Bennett and Lenski 1993, Kolss et al. 2009, Dhar et al. 2011), and about the trade-offs and constraints of adaptations (Lenski 1988). It has provided parameters for estimating population genetics (Halligan and Keightley 2009) and allowed to test evolutionary theories (Beaumont et al. 2009). Despite the great success of experimental evolution there are limitations to the approach, and caution is required when drawing conclusions from the findings of such studies. Particular evolutionary processes might be too slow to be observed within the possible time frame of an evolutionary experiment. For example, the phenomenon of speciation has never been observed to a satisfying degree of distinction (Coyne and Orr 2004). Moreover, evolutionary experiments are prone to contamination which may lead to wrong conclusions (Houle et al. 1994). Finally, the exceedingly high selection pressure (usually only a single factor at a time) and the often small initial population sizes make it more likely that recessive alleles or genes with patterns of pleiotropy are selected, in contrast to nature where selection acts on different alleles at the same time with lower or no adverse pleiotropic effect (Hillenmeyer et al. 2008). As a consequence, such experiments may, e.g. overrate the consequences of evolutionary trade-offs (Kawecki et al. 2012).

Despite its limitations and caveats, experimental evolution using microbes nevertheless represents an exciting and powerful approach to test fundamental

questions in biology. Technological advances, especially in the field of genomics, have further expanded the insight in the molecular mechanisms of evolution, and they have expanded the possible applications of experimental approaches to areas that range from molecular biology to the study of global change (Collins et al. 2006, Lee et al. 2007)

1.5 Bacterial genome evolution and adaptation

For many decades bacteria have been considered as mere containers for genes that evolve due to arbitrary mutations. Novel sequencing technologies have made available a high number of bacterial genomes and have thus opened the way for large-scale comparative analyses of genome content, structure and driving forces of genome evolution (Cordero and Polz 2014). These studies have revealed that microbial genomes have a great variability in size, are highly dynamic and featuring a continuous gain and loss of genetic elements. The main characteristics of bacterial genomes are a simple architecture, high density of genes and a size range from as small as 0.05 Mbp up to 10 Mbp (Koonin and Wolf 2008). The variability in genome size, to some extent, is correlated with the environment of origin: obligate symbionts have the smallest and free-living soil bacteria the largest genomes (Dini-Andreote et al. 2012). Therefore, environmental pressure either leads to an expansion or shrinkage of genomes (Fig.4). Due to the relationship between bacterial genome size and number of genes, a reduction of the genome size is always related to loss of functions (Ochman 2005). In obligate symbionts, essential substrates must be available from the environment to compensate for lost functional capabilities. A plant or animal host offers bacteria a stable environment to bacteria and provide a variety of compounds. This renders genes that were formerly crucial for a free-living life style unnecessary and consequently allows for (and even promotes) the shrinkage of the genome (Klasson and Andersson 2004). However, a gene becoming expendable does not necessarily lead to the elimination of the corresponding sequence from the genome. For example many eukaryotic genomes contain large stretches of “junk DNA” to which only hypothetical functions could have been assigned (Ohno 1972). Moreover, it has been suggested that many annotated genes in bacteria might in fact be pseudogenes and

therefore the coding potential and compactness of many bacterial genomes might be overestimated (Ochman and Davalos 2006).

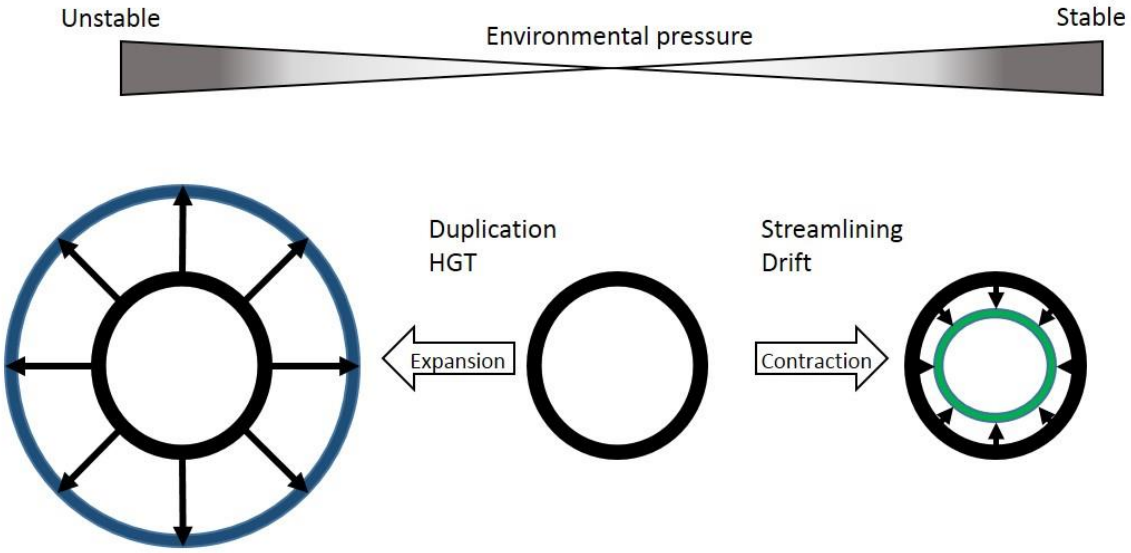

Fig. 4: Influence of environmental conditions on bacterial genome evolution. Modified figure from (Dini-Andreote et al. 2012).

Nevertheless, compared to eukaryotes, bacterial genomes are conspicuously compact. A possible explanation for this lack of DNA with no functional assignment would be a mutational bias towards deletion (Mira et al. 2001). Although such a bias would provide an explanation why prokaryotic genomes are condensed and gene rich, the underlying mechanisms are still unclear. Two processes are considered to be responsible for the decay of genome content: accumulation of mutations, leading to a slow erosion of the respective gene, and deletions of large spans of DNA. Nilson et al found that cultivation of *Salmonella typhimurium* under constant environmental conditions led to several large scale deletions of up to 200 kbp (Nilsson et al. 2005). Deletions of this kind often occur at homologous repeats via RecA-dependent recombination (Klasson and Andersson 2004). However, in that study, only one out of nine deletion endpoints showed homology, whereas the other deletions suggested a RecA-independent mechanism (Nilsson et al. 2005). This laboratory experiment demonstrated that under certain environmental conditions deletions can appear quickly. On the other hand, large excisions bear the risk of accidently deleting essential genes. This would be more detrimental for smaller genomes and therefore would suggest a gene by gene reduction by accumulation of mutations in already reduced genomes (Mira et al. 2001). Altogether, it is likely that both mechanisms in a concerted

manner play an important role in the deletion of genes that are no longer under positive selection.

So far we have discussed the bias towards genome reduction of bacteria in a constant environment, usually endocellular symbionts. These bacteria typically exist in non-recombining small populations. This leads to an accumulation of deleterious mutations, which results in a degenerative process that has been termed ‘Muller’s ratchet’. Thereby, natural selection is displaced by genetic drift that increasingly transforms genes into pseudogenes that are ultimately removed from the genome (Batut et al. 2014). Once lost, genes cannot be regained due to the lack of recombination opportunities with other populations, resulting in a continuous shrinkage of the genome.

However, environmental studies using a host of approaches such as cell counting, isolation, and (meta)genomics (Janssen et al. 1997, Gasol et al. 1999, Biers et al. 2009) have revealed that there are species with very small genomes also among free-living bacteria with large effective population size, both in marine and freshwater habitats, i.e. *Prochlorococcus* (Partensky et al. 1999), *Pelagibacter (Giovannoni et al. 2005), Ac1 Actinobacteria* (Partensky et al. 1999) or *Methylopumilus (Salcher et al. 2015)*. However, the underlying evolutionary process for genome size reduction is very different in endosymbionts and free-living bacteria (Giovannoni et al. 2014). Unlike in symbionts, the major driving force in free-living bacteria is a selection for a cell architecture that minimizes resource requirements. As a consequence, genome signatures are different; the characteristics of free-living streamlined genomes are 1) small and highly conserved core genomes, 2) few pseudogenes, 3) short intergenic spacers and 4) a low numbers of paralogs (Giovannoni et al. 2014). The reason why smaller genome size should be favoured by selection is not totally resolved but several possible explanations exist. Small genomes of free-living bacteria usually show a higher proportion of A and T nucleotides, which would lower the demand for nitrogen (Grzymski and Dussaq 2012), one of the main limiting compounds in the marine environment (Smith 1984). A consequence of small genomes also appears to be a reduced cell size, which in turn increases the surface-to-volume ratio and leads to a more efficient uptake of substrates (Sowell et al. 2008). Finally, a reduced gene content also leads to reduction of the need to regulate genes (Ranea et al. 2005). Therefore

reduced complexity of the environment makes many regulatory proteins superfluous and opens the way for genome reduction.

The above described processes imply that genomes are generally undergoing a process of size reduction. So how do genomes become larger or maintain their size? In fact, genome reduction is not the only successful strategy for adaptation, and specific requirements within a habitat can favour higher genomic complexity and, therefore, larger genomes (Giovannoni et al. 2014). Nevertheless, the question of how genomes increase in size is still not totally resolved. One possible explanation might be the ability of bacteria to exchange genetic material among each other (Koonin et al. 2001) or incorporate it directly from the environment. It has also been shown that phages contribute a substantial part to the lateral acquisition of DNA to their bacterial hosts (Canchaya et al. 2003). Indications of virus mediated virulence factors were found specifically in pathogens (Nakayama et al. 2000, Baba et al. 2002). Furthermore, there are indications that larger genomes are more affected by purifying selection than smaller ones which prevents gene erosion (Koonin and Wolf 2008).

As a stress response to challenging environmental conditions and high mortality rates, genome modifications can lead to an enhanced selection for hypermutator strains (Bjedov et al. 2003). Elevated mutation rates in these strains are usually caused by an increased misincorporation of the DNA polymerase and the abolishing of proofreading and mismatch repair (Bridges 2001). This can be due to an initial damage either in the DNA repair or the SOS mutagenesis systems, e.g. induced by reactive oxygen species or UV (Baharoglu and Mazel 2014). In small populations, where the number of mutations limits evolutionary rates, elevated mutation rates can be useful to acquire a beneficial mutation that helps to overcome the stress induced by the environment, for example the presence of antibiotics (Miller et al. 2002) or lack of essential nutrients (Sniegowski et al. 1997).

Besides all the above described processes, several other genome-affecting mechanisms have been discovered during the last decades that also allow bacteria to react rapidly to environmental changes without influencing the genetic content. Quick adaptations to environmental changes are usually handled by gene regulation, thereby either silencing or enhancing of DNA by regulatory proteins (McAdams et al. 2004). A more complex mechanism to influence transcription is represented by the methylation of specific DNA loci, a process referred as epigenetic regulation (Casadesús and Low

2006). One example of such epigenetic regulation is phase variation, where bacteria can switch rapidly between two phenotypes by altering the methylation patterns of certain genes (van der Woude and Baumler 2004).

2 Aims of the thesis

Microbial predator-prey interactions have been intensely studied in the past. The focus of experimental investigations in model systems has so far been predominantly ecological, e.g. on the induction of morphological changes (Justice et al. 2008). However, the evolutionary consequences of these interactions are only poorly understood, and have been mainly studied in systems of bacteria and phages (Lenski and Levin 1985, Bohannan and Lenski 2000, Buckling et al. 2006). The aim of this thesis was to obtain a better understanding of evolutionary trajectories in predator-prey interactions of heterotrophic nanoflagellates and bacteria. We used a model predator-prey system and an experimental evolution set-up to elucidate the consequences of enhanced predator pressure and nutrient limitation on the evolution of a phenotypically plastic bacterial isolate.

The first objective was to analyse the morphological and physiological adaptations of bacterial strains in a long-term evolution experiment featuring altered substrate levels and concomitant predation pressure.

The second objective was to find genomic changes related to adaptations observed in the evolution experiment. For this purpose we sequenced the genome of the ancestor and evolved strains and analysed their differences. In addition, we developed a competition assay to compare fitness of the ancestor and the evolved strains.

The third objective developed from an observation made during the previous investigation: All strains isolated from the long-term evolution experiment had lost their ability to sense the presence of predators. We thus hypothesized that the maintenance of predator sensing in this strain was dependent on a fluctuating presence of the predator, whereas its continuous presence or absence would select against inducible phenotypic plasticity.

3 Manuscript 1: Long-term evolution experiment

3.1 Introduction to Manuscript 1

In our first study we found rapid adaptations to the set experimental conditions. The strain evolved without predator drastically reduced its antipredator behaviour, i.e. the formation of aggregates. This development was triggered by two environmental factors, nutrient restriction and constant conditions. The reduction of aggregate formation due to nutrient restriction can be best explained by the additional costs for the formation of exopolymeric substances, the basic adhesive element of the aggregate, as illustrated by lectin staining (Fig. 5). Moreover, cells in the centre of aggregates might face bottlenecks with respect to substrate diffusion, leading to so-called intraspecific 'shadow competition' (Lubin et al. 2001) . Thus, if aggregates do not have a beneficial effect on population growth other than defence in a particular growth scenario, they might have a too high cost to benefit ratio in the absence of a mortal threat for the free cell subpopulation, leading to a re-allocation of resources to different processes. The other experimental factor, the constant environment, has been shown on theoretical grounds to have a detrimental effect on phenotypic plasticity (Yamamichi et al. 2011) and eventually also on genome complexity (Lee and Marx 2012). Bacteria from active sludge that form filaments or flocs under predation pressure

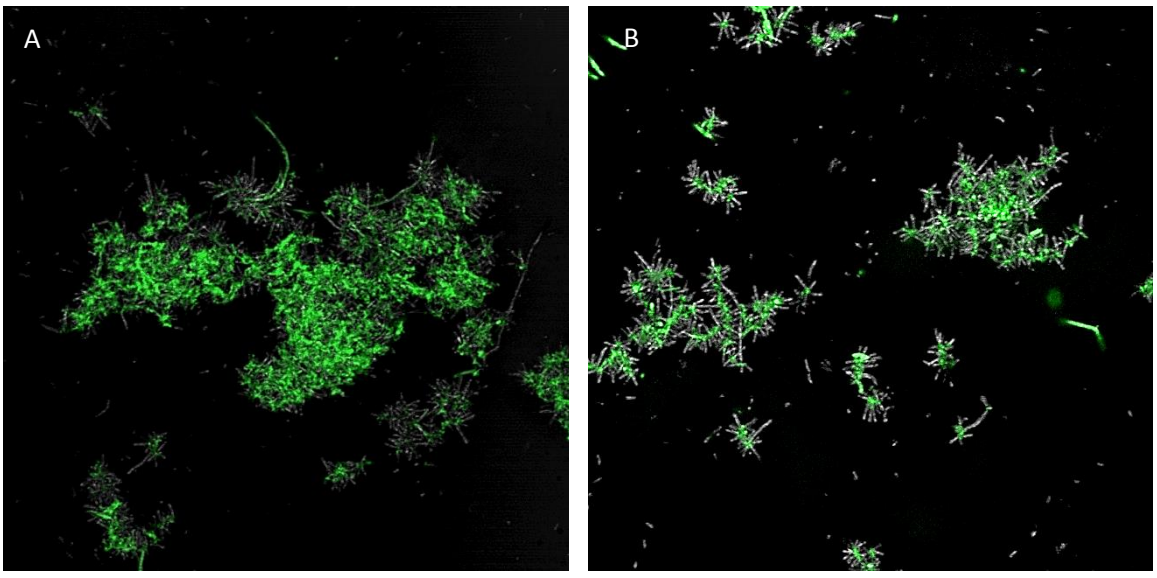

Fig. 5: Lectin staining of strains evolved for 16 growth cycle with the predator. (A) Matrix of an aggregate stained with the lectin AAL specific for fucose and (B) with the lectin PHA-E specific for galactose and N-acetylglucosamine.

in their natural habitat, lost these defence traits in the absence of a predator already after several propagations (Güde 1979).

The reduction of aggregate formation in strains evolved without predator was accompanied by a more rapid growth of single cells and more efficient usage of a variety of single substrates. Growth curve and phenotypic microarrays revealed that strains evolved without predator could grow to higher optical densities on the same quantities of substrate than the ancestor and the strains evolved with predator. For the strains evolved with predator we observed a continuous increase in aggregate formation over the course of the experiment. This suggests an adaptation to the constant predation pressure on free cells. Using feeding rate data of *Poterioochromonas* sp. strain DS from the literature (Boenigk and Arndt 2002), we estimated that approximately 80% of the planktonic bacteria were reduced by grazing of flagellates during each growth cycle already 24h after inoculation. The best option to survive such high mortality was thus to predominately proliferate within the inedible aggregates. Quantification of proteins revealed that around 50% of the total bacterial biomass was found in aggregates, whereas this fraction was less than 8% in the strains evolved without predator.

Our initial interpretation of growth was based on counts of cells and aggregates. Since it was not possible to estimate the numbers of cells within aggregates by direct counting or flow cytometry, we used optical density as a means of assessing the growth of total bacterial population. Optical density measurements of strains evolved with predators usually showed the lowest values. While we initially interpreted these results as an indication of the costs caused by the extended formation of aggregates, later experiments (described in Manuscript 2) revealed an inadequacy of photometric methods to determine and compare the total biomasses of strains that form different amounts of aggregates and biofilms.

3.2 Manuscript 1

Predation on a bacterial strain outweighs substrate limitation in selecting for defence over growth optimized morphotypes

Predation on a bacterial strain outweighs substrate limitation in selecting for defence over growth optimized morphotypes

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Running Title: evolutionary adaptation to oligotrophy and predation

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Abstract

Bacteria are continuously introduced into aquatic habitats from other biomes, where they are exposed to, both, low substrate concentrations and high protistan grazing. While the former selects for the optimization of growth efficiencies, the latter favours traits that reduce predation mortality, such as the formation of large, inedible cell aggregates or microcolonies. Such protected morphotypes often convey a growth disadvantage. Microbes are thus faced with a trade-off between growth and defence traits, which may be realized by a genotypically fixed division into phenotypic subpopulations or by physiological acclimation. We set up an evolutionary experiment with a freshwater isolate that increases aggregate formation if exposed to supernatants from a predator-prey co-culture. We hypothesized that oligotrophic conditions would lead to a reduction of the aggregated subpopulation, and that the concomitant presence of a bacterivorous flagellate might partially or completely rescue this phenotype. After 26 (1-week) growth cycles either with (P+) or without (P-) predators, bacteria had evolved into strikingly contrasting phenotypes. Strains from P- had almost completely lost the aggregated subpopulation, and had gained in fitness, both at the original rich growth conditions and on a number of single substrate sources. By contrast, isolates from the P+ treatment constitutively formed elevated proportions of defence morphotypes, but exhibited reduced metabolic diversity. Moreover, all evolved strains had completely lost phenotypic plasticity, as has been predicted for bistable populations at constant environmental conditions.

Keywords: Experimental evolution, predator-prey interactions, oligotrophy, aggregate formation

Introduction

Early microbiologists assumed that there was “no special and characteristic class of 'water bacteria,' but germs […] may at times find their way into water.” (Ward & Whipple, 1918). While this proposition has been largely refuted in its extreme (Newton *et al.*, 2011), freshwater realms do at least transiently harbour numerous bacterial taxa from other habitats, e.g., mass effects such as terrestrial run-off maintain a continuous influx of allochthonous microbial species (Ruiz-González *et al.*, 2015). It is likely that microbes introduced into freshwaters do not all perish, and some might persist or even thrive in specific niches such as biofilms or sediments.

The lacustrine environment poses great challenges for microbial growth and survival. In most natural waters labile organic compounds such as sugars or amino acids are only available at nanomolar concentrations (Kirchman, 2003). While there are substrate-rich microniches such as suspended organic aggregates (Simon *et al.*, 2002), they occupy a relatively small volume of the total habitat space, thereby lending importance to a free-living life style allowing to transverse large distances between patches of high productivity (Stocker *et al.*, 2008). In the extreme, this has led to the success of entirely planktonic oligotrophy specialists with highly efficient substrate uptake systems (Salcher *et al.*, 2011). Other typical freshwater taxa have maintained a dual strategy of planktonic and surface-attached subpopulations, but are well-adapted to grow at low levels of organic compounds (Kasalicky *et al.*, 2013).

Besides such bottom-up limitations, freshwater microbes suffer high mortality rates from protistan grazers, most prominently from bacterivorous nanoflagellates (Pernthaler, 2005). These predators can control total bacterial abundances and activity (Del Giorgio *et al.*, 1996), and their foraging activity is crucial for the recycling of limiting nutrients (Caron *et al.*, 1988). Protistan grazing is strongly size-selective (Šimek & Chrzanowski, 1992), thereby disproportionally affecting the larger sized cells of copiotrophic bacteria introduced from more productive environments. Bacteria have responded to flagellate predation by evolving various defence strategies (Hahn & Höfle, 2001; Jousset, 2012). Morphological adaptations include the development of a permanently small or large cell size irrespective of growth state (Pernthaler *et al.*, 2004; Salcher *et al.*, 2011), and protection of grazing-resistant subpopulations can be mediated via the formation of large protist-inedible cell aggregates or microcolonies

(Hahn *et al.*, 2004a; Blom *et al.*, 2010a). Such adaptations are believed to come with a fitness cost. For example, cells located within flocks experience reduced diffusive flux at simultaneously high densities, leading to a growth disadvantage reminiscent of intraspecific 'shadow' competition (Lubin *et al.*, 2001). Therefore, it is advantageous that defence mechanisms can be induced if needed, e.g., if the development of inedible morphotypes or cell aggregates is stimulated by a signal from the predator (Corno & Jürgens, 2006; Blom *et al.*, 2010b).

Sphingobium spp. and related genera are consistently found both, in the pelagic zone of freshwaters, and attached to organic particles (Bizic-Ionescu *et al.*, 2015). Furthermore, it was shown that *Sphingobium* spp. is prevalent in treated drinking water, resistant to a wide range of antibiotics and therefore under suspicion of being a reservoir for antibiotic resistance for other bacterial species (Vaz-Moreira *et al.*, 2011). The freshwater bacterial isolate *Sphingobium* sp. Z007 responds to the presence of a bacterivorous nanoflagellate with enhanced formation of flocks, presumably aggregates (Blom *et al.*, 2010a). Supernatants of a predator-prey co-culture also induce this behaviour, indicating the involvement of an infochemical (Blom *et al.*, 2010b). While the strain has been isolated from water samples in the central area of Lake Zurich, its closest relatives from habitats such as soil or the rhizosphere (Pal *et al.*, 2005). It, thus, most likely represents a transient inhabitant of freshwaters with some features being beneficial for its extended survival, but without stable long-term adaptation to this environment. Consequently, this bacterial strain might serve as a model to investigate evolutionary trade-offs between optimization for growth at oligotrophic conditions and defence against predation mortality.

We set up an evolutionary experiment with *Sphingobium* sp. Z007 and the bacterivorous nanoflagellate *Poterioochromonas* sp. strain DS. Bacteria were grown at oligotrophic conditions in semicontinuous culture (weekly re-inoculations) with and without predators for 29 (1-week) growth cycles. We expected that both, oligotrophy and predators would profoundly affect the aggregation of bacteria, albeit in contrasting ways. Specifically, we hypothesized that aggregate formation would be highly disadvantageous and eventually reduced during growth at low substrate concentrations, but that the presence of nanoflagellates would partially or completely rescue this behaviour. Additionally, we examined if the permanent presence or

absence of flagellates under severely growth-limiting conditions would affect the predator sensing abilities of *Sphingobium* sp. Z007.

Materials and Methods

Microbial strains, cultivation conditions

Sphingobium sp. strain Z007 (EMBL 16S rRNA gene accession number, FN293045) was originally isolated from the surface water of mesotrophic Lake Zürich in 2006 (Blom & Pernthaler, 2010). It is a gram negative, rod shaped bacterium with the ability to form large aggregates / microcolonies. Stock cultures of this strain were preserved at -80°C in 50% glycerol (50%, Sigma). For the experiments it was first regrown in nutrient rich DSMZ 7 medium (yeast extract 1 g L^{-1}; glucose 1 g L^{-1}; peptone 1 g L^{-1}; German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany) in 300 ml Erlenmeyer flasks at 18°C in the dark. Bacteria were transferred into fresh medium 72 h before starting the experiments. During the evolution experiment strains were isolated at different time points. These isolates were also preserved at -80°C in 50% glycerol and regrown in DSMZ 7 medium as described above for further experiments. Axenic cultures of the bacterivorous mixotrophic flagellate predator *Poterioochromonas* strain DS were used for the experiments (Blom *et al.*, 2010b). Stock cultures of the flagellates were maintained in nutrient rich '*Ochromonas*-medium' (yeast extract 1 g L^{-1}; meat extract 1 g L^{-1}; glucose 1 g L^{-1}; peptone 1 g L^{-1}; Culture Collection of Algae at the University of Göttingen, Germany) at 18 ° C in the dark, and flagellates were fed weekly with heat killed bacteria from a different species (*Flectobacillus major* DSMZ 103, 1 x 10^7 cells ml^{-1}; preincubated at 70°C for 2h). Prior to the experiments flagellate cultures were inoculated into fresh medium and starved for 72 h.

The evolution experiment was carried out in nutrient-poor oligotrophic medium. Artificial lake water (ALW), a mix of inorganic nutrients and trace elements (Zotina *et al.*, 2003), was amended with the substrates of the DSMZ 7 medium at 1:1000 dilution (yeast extract 1 mg L^{-1}; glucose 1 mg L^{-1}; peptone 1 mg L^{-1}). All other experiments were performed in DSMZ 7 medium. For isolation of strains on plates, DSMZ 7 medium was amended with 16.4 g L^{-1} of agar.

Quantification of bacteria and flagellates

Samples for flow cytometry were stained with DAPI (4,6-Diamidino-2-phenylindole, Serva, 1µg ml^{-1}) for 30 minutes in the dark and analysed using an InFlux V-GS cell sorter (Becton Dickinson Inc., San Jose, CA). A UV Laser (60 mW, 355 nm; CY-PS; Lightwave Electronics) was used for detection of DAPI fluorescence, and a blue laser (200 mW, 488 nm; Sapphire; Coherent Inc.) for scattered light and auto-fluorescence of flagellates. If required, samples were diluted with deionized water prefiltered with a 0.2-µm-pore-size filter to avoid particle coincidence. Data analysis was carried out with an in-house custom software (J. Villiger & J. Pernthaler, unpublished). Single cells of *Sphingobium* sp. strain Z007 were identified using side scattered light (SSC) versus DAPI fluorescence (431 nm). *Poterioochromonas* sp. strain DS was identified using SSC versus green fluorescence (531 nm). Flagellates were gated and excised from the dataset. Aggregates were operationally defined by fluorescence and scatter properties equal to or higher than that of *Poterioochromonas* sp. strain DS (Blom *et al.*, 2010b).

Evolution experiment

The evolution experiment was carried out with bacterial cultures (treatment P-) and co-cultures of bacteria and flagellates (treatment P+) by serial propagation under semi continuous conditions in ALW medium. The experiment was run for 200 days (approximately 29 weeks) in 300 ml Erlenmeyer flasks in a volume of 100 ml medium at 18°C in the dark. Five batch cultures were inoculated only with *Sphingobium* sp. strain Z007 (initial densities 1.5 x 10^6 cells ml^{-1}), or with *Sphingobium* sp. strain Z007 (initial densities 1.5 x 10^6 cells ml^{-1}) and *Poterioochromonas* sp. strain DS (initial densities, 1.5 x 10^3 cells ml^{-1}). All cultures were re-inoculated weekly. For this purpose, ten millilitres of the cultures or co-cultures were added to 90 ml of fresh ALW medium. Subsamples of 1 ml were taken at three time points (72, 120, and 168 h) after addition of new medium and fixed with glutaraldehyde (final concentration, 2.5%) to determine the abundances of bacterial single cells, aggregates, and flagellates. Samples were stored at 1°C until they were analysed by flow cytometry (usually within 24 to 48 h).

Starting from day 18, subsamples were taken biweekly from each of the ten Erlenmeyer flasks and plated on DSMZ 7 medium agar plates, to separate bacteria from flagellates (for the co-culture treatments), and to check for possible

contamination. After three days, bacterial colonies were picked from the plates, suspended in 1 ml glycerol (50%, Sigma), and stored at -80 °C until further analyses. These isolates are denominated according to treatment (P+ or P-) and the number of growth cycles (weeks) they spent in the evolution experiment (e.g. 7, 16, 26 cycles).

Growth curves and aggregation of evolved strains

Growth curves were determined from bacterial strains that had been isolated from 10 time points of the evolution experiment. Colonies were suspended in DSMZ 7 medium to a final concentration of approximately 1 x 10^5 ml^{-1}. Five strains, each derived from a different experimental replicate, were analysed per treatment (P+ or P-). Bacterial growth was measured as optical density at 600 nm (OD_{600}) in a absorption plate reader (Spectra Max 190, Molecular Device Corporation, Sunnyvale, CA) every 60 min for 72 h. Mean values and standard errors of all replicate measurements were calculated for each time point. The growth curve of every strain and of 3 separate colonies of the ancestor strain were measured in triplicate. The numbers of free cells and aggregates of 5 isolates from all 10 time points was determined by flow cytometry (see below).

Biofilm formation

To determine the ability of evolved *Sphingobium* strains to form biofilms isolates from both treatments from growth cycle 7, 16, and 26 were grown in DSMZ 7 medium for five days at room temperature. Bacteria were re-inoculated into DSMZ 7 medium to a final concentration of approximately 2 x 10^5 ml^{-1}, transferred into a 96-well plate, and incubated in the dark at 18°C. All isolates were measured in triplicate. After 72 h of growth all wells were incubated for ten minutes with 40 µl of a 0.1% crystal violet solution (in H_2O, room temperature). Subsequently, the liquid phase was discarded and the wells were washed three times with PBS buffer (50 mM; pH 7.4). After drying for 15 min at room temperature 200 µl of 95% ethanol were added to each well to solubilize the precipitated crystal violet. The solvent was mixed well by pipetting, incubated for 15 min, and absorption was measured at 600nm.

Direct predator contact experiments with evolved strains

Sphingobium strains were co-cultivated with *Poterioochromonas* sp. strain DS following the setup described by Blom et al. (Blom *et al.*, 2010a). Experiments were carried out with the ancestor strain and with strains obtained from three time points (7, 16, 26 growth cycles) of both treatments of the evolution experiment. Individual sets of batch culture experiments performed using three evolved strains isolated from different experimental vessels, or in triplicate for the ancestor strain. Bacteria and flagellates were inoculated at initial densities of 1.0 x 10^6 ml^{-1} and 1.0 x 10^3 ml^{-1}, respectively. Control experiments were performed without addition of predators. All experiments were carried out in 300 ml Erlenmeyer flasks in a final volume of 50 ml DSMZ 7 medium at 18°C in the dark. Subsamples of 1 ml were taken every 24 h until the end of the experiment (72 h) and fixed with glutaraldehyde (final concentration 2.5%). Samples were stored at 1°C until further analysis by flow cytometry (within 24 to 48 h).

Experiments with supernatants

Bacterial strains isolated after 26 growth cycles from both treatments of the evolution experiment were tested for their ability of induced aggregate formation by a chemical cue. The setup for these experiments basically followed the one described in Blom et al (Blom *et al.*, 2010b). Briefly, the *Sphingobium* sp. Z007 ancestor strain was co-cultivated with *Poterioochromonas* sp. strain DS for 120 h (initial densities of bacteria and flagellates, 1.0 10^6 ml^{-1} and 1.0 10^3 ml^{-1}) in DSMZ 7 medium. Subsequently, cultures were centrifuged two times for 15 min at 9000 × g under sterile conditions. The pellet was subsequently discarded and the supernatants were frozen at -20 °C for at least one hour to destroy any remaining flagellate cells. Immediately after thawing, the supernatants were filled in 24-well plates and inoculated with 1.0 × 10^6 ml^{-1} of the evolved strains (final volume, 2.0 ml). Control treatments were supernatants of *Sphingobium* sp. Z007 cultures without addition of flagellates. Subsamples (500 µl) were taken after 48 h, fixed with glutaraldehyde (final concentration 2.5%), and analysed by flow cytometry.

Lectin staining of aggregates

For the staining of cell aggregates with fluorescently labelled lectins (Neu *et al.*, 2001) a bacterial strain was used that had been isolated after 16 growth cycles from treatment P+. Bacteria from the -80°C stock cultures were grown for 120 h in DSMZ 7 medium. The cells in the aggregates were counter-stained with the nucleic acid specific dyes SYTO 9 or SYTO 60 (Molecular Probes). Seventy seven fluorescent lectins labelled either with the fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) were dissolved in a solution of 0.1 mg ml^{-1} in filter-sterilized (0.2 µm) H_2O . The samples (500 µl of bacterial culture) were mixed with 500 µl of a solution with fluorescent lectins and stained for 20 min in the dark at room temperature. Subsequently, the bacterial aggregates were carefully washed three times with deionized H_2O. After the last centrifugation step the bacterial aggregates were suspended in deionized H_2O, transferred to a glass microscope slide and covered with a coverslip for optimal fluidity of the samples. The sample drop volume was adjusted to 20 µl to minimize the floating of bacterial aggregates. Samples were examined under a confocal laser scanning microscope (Leica TCS SP1) equipped with appropriate filter combination for the imaging of dye pairs (TRITC / SYTO 9 or FITC / SYTO 60).

Substrate spectra and growth on single substrates

The *Sphingobium* sp. strain Z007 ancestor and isolates from three replicate treatments evolved for 26 growth cycles with or without flagellates were streaked on DSMZ 7 agar plates and incubated at 18°C in the dark. Colonies were carefully picked after 72h, avoiding nutrient contamination from the agar plates, suspended in 2 ml of ALW, and OD_{600} was adjusted to 0.18 (Cary 3, Varian). 1.5 ml of the suspension was diluted with 13.5 ml of ALW. 100 µl of the diluted suspension was transferred to each well of the PM1 and PM2 substrate array plates (Biolog, USA). The plates were incubated in the dark at 18°C. After 48h of incubation, 1.5 µl of the redox dye A (Biolog, USA) was added, and plates were incubated for another 24h at the conditions described above. Individual wells were then tested for respiratory activity by measuring optical density at 590 nm (OD_{590}) -the absorption maximum of the redox dye- with an absorption microplate reader (Spectra Max 190, Molecular Devices). The integrated OD_{590} value of the negative control wells was then subtracted from each measurement. Only background-corrected OD_{590} values >0.05 were considered as positive growth.

After establishing the substrate spectra qualitatively, we also quantitatively compared growth of the ancestor and evolved strains on a subset of single substrates. For this purpose, the above assays were carried out without the (potentially toxic) redox dye on substrates utilized by all strains. Plates were incubated for 72h at 18°C in an absorption microplate reader (Spectra Max 190, Molecular Devices) that recorded OD_{600} every 15 min. Prior to every read the plate was shaken for three seconds. OD_{600} readings were corrected by the corresponding values of negative controls (bacteria incubated in substrate-free ALW). The length of the lag phase (λ) preceding exponential growth was determined as the period until the first doubling of OD_{600}. The area under the growth curve (AUC) was determined as a proxy of total growth yield.

Statistical analyses

Differences in the numbers of single cells and aggregates between the ancestor and the evolved strains or in the aggregate-to-free-cell ratios were analyzed by two-sided Dunnett's tests. For the comparison of growth yield on different substrates and the duration of the lag phase, the results from individual substrates were pooled into the classes amino acids (n=9), carbolic acids (n=5), dipeptides (n=3), monosaccharides (n=6) and oligosaccharides (n=4). First, a 2-way ANOVA with the dependent variables strain type and substrate class was performed. After establishing that the two factors as well as their interaction were significant at $P<0.05$, the individual differences between the ancestor and the evolved strains were assessed by Dunnett's tests. In the supernatant experiment we tested for differences between the treatments by Tukey's range tests. All statistical analysis was carried out with SPSS (IBM Corp).

Results

Evolution experiment

The end points of the growth curves of each growth cycle (1 week or 168 h) are depicted in Fig 1. In the P- treatment, the final numbers of free bacterial cells increased from 3×10^6 ml^{-1} to approximately 20×10^6 ml^{-1} after 10 weeks (Fig. 1A), and ranged around $19 \pm 3.7 \times 10^6$ ml^{-1} thereafter. With the exception of one peak after 10 growth cycles (1.8×10^3 ml^{-1}) the numbers of aggregates remained stable in this treatment, at

around 0.2 x 10^3 ml^{-1} (Fig. 1B). In co-cultures of *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS (treatment P+) the numbers of flagellates stabilized at around 5.6 ±1.6 x 10^3 ml^{-1} already after 2-3 growth cycles, and reached somewhat higher levels towards the very end of the experiment (Fig. 1A). The abundances of free bacterial cells in P+ initially dropped to 0.05 x 10^6 ml^{-1}, but increased gradually over the course of the experiment, to eventually 1.4 x 10^6 ml^{-1}. The number of aggregates in the bacteria-predator co-culture was always higher in P+ than in P- (around 1.8 x 10^3 ml^{-1}). It transiently increased in the period between 8-14 growth cycles, and again from week 22 until the end of the experiment (3.1 ±0.69 x 10^3 ml^{-1}, Fig. 1B).

Growth curves of isolates

After a lag phase of about five to six hours the ancestor strain showed steep exponential growth until an OD_{600} of 0.52 after about 27 h (Fig. 2A). Thereafter, OD_{600} slowly declined to <0.4. For the sake of clarity, only the growth curves of evolved isolates from 3 time points are depicted in Fig 2A. All evolved strains had a longer lag phase on rich media. However, strains showed clearly contrasting growth patterns related to their treatment of origin, with little differences between isolates from different time points. In P- strains, exponential growth was slower than in the ancestor, with strain 178 P- not even reaching the stationary phase within the 70 hours of the experiment. However, the OD_{600} of all P- strains were higher than of the ancestor strain. The isolated strains from the P+ treatments grew most slowly, and only reached OD_{600} values of up to 0.32.

Biofilm formation, partitioning of populations into free cells and aggregates

Sphingobium sp. strains that were isolated after 7 growth cycles from either treatment of the long-term evolution experiments showed increased biofilm formation compared to the ancestor strain (Fig. 2B). By contrast, isolates from last time point (26 growth cycles) clearly differed in their response according to their respective origin: there was significantly lower crystal violet precipitation in samples of strains from the P- treatment, whereas biofilm formation remained higher than that of the ancestor in the corresponding strains from the P+ treatment.

Up to four times more free cells could be detected in isolates from the predator-free environment (P-) than in bacterial strains that were isolated from treatments under predation pressure (P+) (Fig.3A). These differences were already highly significant after 7 growth cycles. In turn, isolates from the P+ treatment always tended to form aggregates (Fig.3B). Enhanced aggregate formation became highly significant in strains from the P+ treatment isolated after 12 growth cycles and later ($P<0.05$). After 26 growth cycles the number of aggregates in strains from P+ was >50 times higher than in the corresponding P- strains.

Lectin staining

Six of the 77 tested lectins showed strong binding signal either to the extracellular polymerous substances (EPS) or to the surfaces of aggregated bacterial cells (Table 1). The fucose specific lectins mainly bound to the EPS matrix and only to a minor extend to the glycocalix (Fig. 4 A,B). Lectins with a specificity for N-acetylglucosamine (WGA) and N-acetylgalactosamine (HPA) were found to exclusively stain cell surface compounds (Fig.4C) or the matrix (Fig. 4D), respectively. Two distinct bacterial cell morphotypes, filaments and short rods, could be distinguished within the aggregates (Fig.4A), whereas free-living cells were always rod-shaped (data not shown). Interestingly all lectins except for HPA were found to preferably bind to the filaments, sometimes exclusively so (WGA, Fig. 4D).

Phenotype microarrays, growth on single substrates

Sphingobium sp. strain Z007 showed unambiguous strong growth (i.e., in all 3 technical replicates) on 28 substrates of the PM1 and PM2 microarrays, and growth on another 14 substrates was observed in 1 or (mostly) 2 replicates (Table 2). Highest OD_{590} was observed on glutamine, glutamate, citric acid, (methyl) pyruvate, and on various mono- and oligosaccharides. In most instances, all 3 strains isolated from different replicates of the evolution experiment showed a similar positive or negative response to the offered substrates, but there were several instances (4 in P+ and 3 in P-) when one of the strains diverged. More frequently, there were differences between the two technical replicates of individual isolates, likely due to a weak growth response at the lower detection limit of the method. In contrast to the ancestor, none of the

evolved strains visibly grew on lactic acid. In addition, all isolates from the P+ treatments apparently lost the ability to grow on 4-hydroxy benzoic acid and D-mannose. By contrast, the P- strains showed growth on substrates where no growth of the ancestor could be detected (e.g. on the exotic monosaccharides D-Fucose and L-Lyxose).

All tested strains showed respiratory activity on a subset of 30 substrates. For 27 of these substrates, positive growth could also be unequivocally established without the addition of the redox dye, i.e., by direct measurements of OD_{600}. These substrates were, therefore included in the subsequent quantitative comparison of growth parameters (Fig 5). Strains from the P- treatment showed significantly higher growth yield than the ancestor in all but one substrate class (carbolic acids) (Fig. 5A). Incidentally, this was also the only category where P+ strains showed less growth than the ancestor. A significant reduction (P- strains) or prolongation (P+ strains) of the duration of the lag phase was also apparent in some of the substrate classes (Fig. 5B).

Aggregate formation in the presence of predators and in supernatants

The ratio of aggregates to free cells (r_A) in the ancestor strain was approximately 40 times higher under flagellate predation than in pure culture, and between 5 to 10 times higher in the evolved strains (data not shown). Strains evolved under predation had significantly higher r_A than the ancestor when grown together with flagellates, whereas the opposite was the case for those strains from the P- treatment that had been isolated after 16 and 26 growth cycles (Fig 6A). Only the ancestor strain responded to supernatants from a predator-prey co-culture with a significant increase of r_A, as compared to supernatants from a bacterial culture only (Fig. 6B).

Discussion

The facultative oligotrophic life style

Bacteria rapidly respond to a transfer to more productive environments both by phenotypic change and genotypic adaptation. This is amply illustrated by the procedure of obtaining and maintaining pure cultures: while many oligotrophic

freshwater bacteria are initially not able to form colonies on solid rich media, their cultivability can be substantially enhanced by prior acclimatization in substrate amended liquid culture (Hahn *et al.*, 2004b). Moreover, prolonged periods of starvation may lead to the transformation of obligate into facultative oligotrophs (Schut *et al.*, 1993). By contrast, the adaptations of bacteria upon transition from richer niches to suboptimal substrate conditions that go beyond physiological acclimation are much less understood. Cultivable microbes with 'copiotrophic' growth forms are ubiquitously present in aquatic microbial assemblages and can be isolated even from remote oceanic sites (Schut *et al.*, 1997). Such bacteria will sporadically find productive microniches, e.g., on suspended particulate organic matter (Grossart *et al.*, 2003). At other times they may be pressed to explore the pelagic zone despite low or patchy substrate availability, suggesting that they should maintain advantageous metabolic features such as motility (Grossart *et al.*, 2001) or chemotactic sensing (Stocker *et al.*, 2008). This condition markedly differs from the 'starvation' type of metabolism and has been referred to as the 'hungry' mode of bacterial growth at limiting supply of substrates or nutrient (Ferenci, 2001).

Effects of oligotrophic growth conditions

Despite its prolonged propagation in pure culture at rich conditions, *Sphingobium* sp. strain Z007 is a facultative oligotrophic strain capable of growth at 1000 fold dilution of a complex medium (Fig. 1). Our experimental setup was a sequential batch culture. Thus, bacteria did not experience constant growth conditions, but rather repeatedly moved through distinct phases: the initial period of density-independent growth at low substrate concentrations (early exponential phase) might be analogous to continuous cultivation at C-limiting conditions , whereas the increasingly substrate-limiting conditions during the late exponential to early stationary phase eventually led to a transition into non-growth. Thus, adaptation pressure on *Sphingobium* was probably more multifaceted than in a constant environment. Our oligotrophic growth conditions thus might have led to a selective trade-off, as described for *E.coli* evolving at oscillating 'feast-or-famine' conditions (Ying *et al.*, 2015). However, in contrast to the setup of that study, we maintained bacteria 'hungry' (Ferenci, 2001) and avoided extended periods of starvation between cycles. This might

be the reason why we did not observe a growth disadvantage of the evolved strains in rich media (Ying *et al.*, 2015).

On the contrary, the strains from the P- treatment grew considerably more efficiently on rich media than the ancestor, as deduced from OD measurements (Fig. 2), albeit at the price of a prolonged lag phase. Interestingly, this apparent difficulty to acclimatize to rich growth conditions was only observed on a complex substrate mix, whereas growth in the P- strains initiated significantly earlier than in the ancestor on three out of five types of single carbon sources (amino acids, monosaccharides, oligosaccharides; Fig. 5). This might be interpreted in the context of the model for facultative oligotrophic growth by Egli (Egli, 2010): Slowly growing *E.coli* cultivated on glucose maintained 'multivory', i.e. their ability to transport and metabolize a range of other substrates, and consequently their preparedness for substrate co-utilization and/or instantaneous switching. It is thus conceivable that the evolved *Sphingobium* isolates also improved in their 'latent' ability to immediately utilize a variety of single C sources. The closely related oligotrophic marine species *Sphingopyxis alaskensis* virtually showed no lag phase if slowly-growing or starving cells were re-inoculated into glucose-rich medium (Eguchi *et al.*, 1996).

Sphingobium sp Z007 strains evolving at oligotrophic growth conditions without predators also underwent other phenotypic adaptations such as significantly higher numbers of free single cells (Fig. 1, 2) and enhanced growth yield on several classes of single substrates (Fig. 5a). These changes were likely more than mere physiological acclimation, since they were manifested at rich substrate conditions by isolates resuscitated from frozen stocks. The observed rather broad upshift in substrate utilization efficiency may not necessarily require the unlikely accumulation of several beneficial mutations. Sub-optimal growth conditions induce a stress response in *E.coli* mediated by the alternative sigma factor RpoS; differences in rpoS expression may profoundly affect bacterial carbon utilization patterns (King *et al.*, 2004) and phenotypic diversity may arise solely by selecting for rpoS mutations in environmental *E. coli* isolates (Chiang *et al.*, 2011). A similar reasoning might be applied to explain the shifted balance of planktonic vs. aggregated subpopulations in evolved *Sphingobium* (Fig. 2): The biofilm phenotype is often induced by the quorum sensing system (Huber *et al.*, 2001), which in turn may be related to the RpoS regulon (Schuster *et al.*, 2004). While at this stage we can only speculate about the molecular mechanisms behind the observed adaptations, it is nevertheless noteworthy that the evolutionary passage

through a bottleneck of oligotrophy rendered evolved *Sphingobium* sp. more efficient also at rich substrate conditions (Fig. 2). Thus, it is conceivable that the transfer to oligotrophic habitats may in fact not be a one-way route for these bacteria, but may conserve or even enhance their competitiveness in more productive niches.

An evolutionary experiment not explicitly regarded as such (Hood & Macdonell, 1987) may in fact be the closest match to our setup: The serial transfer of marine *Vibrio* isolates to liquid media with decreasing substrate concentrations (from g to mg C L^{-1}) induced the dominance of strains with a 'facultative oligotrophic' life style that were able to grow in both, rich and poor media. Interestingly, a small minority of strains from that experiment were unable to grow in rich media. Various mechanisms may give rise to genotypic variability in complex environments even in originally isogenic populations (Barrett *et al.*, 2005; Puentes-Teliez *et al.*, 2013). Since we only sampled 3 clones from each of the 3 treatment replicates for the assessment of growth patterns and phenotypes (Figs. 2, 3, 5), the total extent of evolved genotypic variation in our experiment may have been underestimated. On the other hand, the overall adaptive response of bacteria evolved in sequential batch culture on a complex medium may be rather uniform, notwithstanding clonal genotypic heterogeneity (Puentes-Teliez *et al.*, 2013).

Simultaneous exposure to predation and substrate limitation

The increasing appreciation of contemporary evolutionary processes at quasi-ecological time scales (Schoener, 2011) has stimulated the interest in feedbacks between physiological acclimation or trait selection and organismic interactions such as trophic relationships (Yamamichi *et al.*, 2011). For example, predation has been shown to delay bacterial diversification, as compared to competition (Meyer & Kassen, 2007), and resource availability may affect, both, the formation of defence phenotypes and the stability of predator-prey interactions (Friman *et al.*, 2008),

In our evolutionary experiment, the presence of predators clearly outweighed substrate limitation in determining the instantaneous balance of morphotypes (Fig. 1), and this treatment selected for genotypes (strains) with constitutively enhanced rather than reduced aggregate and biofilm formation (Fig. 2). A prolonged co-cultivation of *Serratia marcescens* and the ciliate *Tetrahymena thermophila* also pushed bacteria towards more grazing-resistant forms, both at low and high resource availability

(Friman *et al.*, 2008). Such defence phenotypes are usually considered to represent a trade-off in terms of growth: Both, the formation of flocs and biofilms requires higher production of extracellular polymeric substances (Hahn *et al.*, 2004a), e.g., polysaccharides (Yang & Kong, 2012), which is a particularly costly investment at substrate-limiting conditions. *Sphingobium* aggregates appeared to be of complex composition rather than the results of chance encounters of free cells, as illustrated by a variety of cellular morphotypes with contrasting surface properties that were revealed by staining with fluorescently labelled lectins (Fig. 4). One might speculate that specific well-differentiated phenotypes, e.g., filamentous forms or microcolonies (Fig. 4D), might have served as the seeds for flock formation, as has been described for aggregates composed of two species (Corno *et al.*, 2013). In any case, the aggregated phenotype likely represented a growth disadvantage and thus mainly functioned as a refuge from flagellate predation (Hahn *et al.*, 2004a).

The over-aggregating strains from the predator-exposed treatments exhibited metabolic disadvantages: they were not able to visibly metabolize a number of single substrates that were readily consumed by the ancestor and the P- strains (Table 2), and there was no indication of a fitness benefit of P+ strains on any of the growth-supporting substrate classes (Fig. 5). A treatment-specific shift in the number of metabolized substrates has also been noted during evolution of *E.coli* on complex media (Puentes-Teliez *et al.*, 2013). The apparent loss of physiological functions and concomitantly enhanced aggregation in *Sphingobium* might be interpreted in terms of antagonistic pleiotropy: A *Pseudomonas fluorescens* genotype that had superior fitness in a particular microniche (the air-water interface) at the same time exhibited restrictions of carbon catabolism, and both phenomena were due to a mutation at the same locus (MacLean *et al.*, 2004).

Trait displacement vs. physiological acclimation

A common feature of all evolved strains irrespective of treatment was the apparent loss of phenotypic plasticity: For one, strains from the P+ treatment constitutively formed high numbers of aggregates in pure culture (Fig. 2), and the presence of predators did not induce this phenotype in strains from the P- treatment (Fig. 6A). Moreover, supernatants of a spent predator-prey co-culture did not result in enhanced aggregation in any of the evolved strains, whereas this was the clearly case

in the ancestor (Fig. 6B) (Blom *et al.*, 2010b). One might envisage that there is a succession of a short-term ecological selection for particular morphotypes in a phenotypically plastic population and a subsequent evolutionary selection within this ecologically favoured subpopulation. For example, the unicellular cyanobacteria *Microcystis* immediately forms grazing-resistant microcolonies in the presence of a bacterivorous flagellate (Burkert *et al.*, 2001). However, these structures are composed of substantially fewer cells than in field samples, and only prolonged (50 d) co-cultivation with a predator leads to the progressive size increase of the *Microcystis* flocks to natural levels (Yang & Kong, 2012).

Theoretical analyses illustrate that the 'specialist' genotype will always outcompete the plastic 'generalist' at constant growth conditions, if there is a fitness cost to environmental perception and conditional physiological adaptation (Yamamichi *et al.*, 2011). As outlined above, growth conditions were probably not entirely constant even in the absence of predators, and the somewhat delayed growth dynamics of bacteria and flagellates (data not shown) suggest that this was even more the case for the P+ treatments. However, the propagation of bacteria from the P+ variants between individual growth cycles always took place when a large part of the total population was situated within aggregates (Fig. 1). Therefore, selection was consistently biased for this growth type, which in turn appeared to be sufficient to favour genotypes with high constitutive aggregate and biofilm formation over the phenotypically plastic ones. It remains to be investigated whether phenotypic plasticity might be preserved in evolving *Sphingobium* sp. Z007 if maintained as suggested by theory (Yamamichi *et al.*, 2011), i.e., at more drastic fluctuations of top-down and bottom up control.

References

Barrett, R. D. H., R. C. MacLean, and G. Bell. 2005. Experimental evolution of Pseudomonas fluorescens in simple and complex environments. American Naturalist **166**:470-480.

Bizic-Ionescu, M., M. Zeder, D. Ionescu, S. Orlic, B. M. Fuchs, H. P. Grossart, and R. Amann. 2015. Comparison of bacterial communities on limnic versus coastal marine particles reveals profound differences in colonization. Environmental Microbiology **17**:3500-3514.

Blom, J. F., K. Horňák, K. Šimek, and J. Pernthaler. 2010a. Aggregate formation in a freshwater bacterial strain induced by growth state and conspecific chemical cues. Environmental Microbiology **12**:2486-2495.

Blom, J. F. and J. Pernthaler. 2010. Antibiotic effects of three strains of chrysophytes (Ochromonas, Poterioochromonas) on freshwater bacterial isolates. Fems Microbiology Ecology **71**:281-290.

Blom, J. F., Y. S. Zimmermann, T. Ammann, and J. Pernthaler. 2010b. Scent of Danger: Floc Formation by a Freshwater Bacterium Is Induced by Supernatants from a Predator-Prey Coculture. Applied and Environmental Microbiology **76**:6156-6163.

Burkert, U., P. Hyenstrand, S. Drakare, and P. Blomqvist. 2001. Effects of the mixotrophic flagellate *Ochromonas* sp. on colony formation in *Microcystis aeruginosa*. Aquatic Ecology **35**:11-17.

Caron, D. A., J. C. Goldman, and M. R. Dennett. 1988. Experimental demonstration of the roles of bacteria and bacterivorous protozoa in plankton nutrient cycles. Hydrobiologia **159**:27-40.

Chiang, S. M., T. Dong, T. A. Edge, and H. E. Schellhorn. 2011. Phenotypic diversity caused by differential RpoS activity among environmental *Escherichia coli* isolates. Applied and Environmental Microbiology **77**:7915-7923.

Corno, G. and K. Jürgens. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. Applied and Environmental Microbiology **72**:78-86.

Corno, G., J. Villiger, and J. Pernthaler. 2013. Coaggregation in a microbial predator-prey system affects competition and trophic transfer efficiency. Ecology **94**:870-881.

Del Giorgio, P. A., J. M. Gasol, D. Vaqué, P. Mura, S. Agusti, and C. M. Duarte. 1996. Bacterioplankton community structure: protists control net production and the propoportion of active bacteria in a coastal marine community. Limnology and Oceanography **41**:1169-1179.

Egli, T. 2010. How to live at very low substrate concentration. Water Research **44**:4826-4837.

Eguchi, M., T. Nishikawa, K. MacDonald, R. Cavicchioli, J. C. Gottschal, and S. Kjelleberg. 1996. Responses to stress and nutrient availability by the marine ultramicrobacterium Sphingomonas sp strain RB2256. Applied and Environmental Microbiology **62**:1287-1294.

Ferenci, T. 2001. Hungry bacteria - definition and properties of a nutritional state. Environmental Microbiology **3**:605-611.

Friman, V. P., T. Hiltunen, J. Laakso, and V. Kaitala. 2008. Availability of prey resources drives evolution of predator-prey interaction. Proceedings of the Royal Society B-Biological Sciences **275**:1625-1633.

Grossart, H. P., T. Kiorboe, K. Tang, and H. Ploug. 2003. Bacterial colonization of particles: Growth and interactions. Applied and Environmental Microbiology **69**:3500-3509.

Grossart, H. P., L. Riemann, and F. Azam. 2001. Bacterial motility in the sea and its ecological implications. Aquatic Microbial Ecology **25**:247-258.

Hahn, M. W. and M. G. Höfle. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. Fems Microbiology Ecology **35**:113-121.

Hahn, M. W., H. Lunsdorf, and L. Janke. 2004a. Exopolymer production and microcolony formation by planktonic freshwater bacteria: defence against protistan grazing. Aquatic Microbial Ecology **35**:297-308.

Hahn, M. W., P. Stadler, Q. L. Wu, and M. Pockl. 2004b. The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria. Journal of Microbiological Methods **57**:379-390.

Hood, M. A. and M. T. Macdonell. 1987. Distribution of Ultramicrobacteria in a Gulf-Coast Estuary and Induction of Ultramicrobacteria. Microbial Ecology **14**:113-127.

Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl. 2001. The cep quorum-sensing system of Burkholderia cepacia H111 controls biofilm formation and swarming motility. Microbiology-Sgm **147**:2517-2528.

Jousset, A. 2012. Ecological and evolutive implications of bacterial defences against predators. Environmental Microbiology **14**:1830-1843.

Kasalicky, V., J. Jezbera, M. W. Hahn, and K. Simek. 2013. The Diversity of the Limnohabitans Genus, an Important Group of Freshwater Bacterioplankton, by Characterization of 35 Isolated Strains. Plos One **8**.

King, T., A. Ishihama, A. Kori, and T. Ferenci. 2004. A regulatory trade-off as a source of strain variation in the species Escherichia coli. Journal of Bacteriology **186**:5614-5620.

Kirchman, D. L. 2003. The contribution of monomers and other low-molecular weight compounds to the flux of dissolved organic material in aquatic ecosystems.*in* S. E. G. Findlay and R. L. Sinsabaugh, editors. Aquatic ecosystems: Interactivity of dissolved organic matter. Academic Press.

Lubin, Y., J. R. Henschel, and M. B. Baker. 2001. Costs of aggregation: shadow competition in a sit-and-wait predator. Oikos **95**:59-68.

MacLean, R. C., G. Bell, and P. B. Rainey. 2004. The evolution of a pleiotropic fitness tradeoff in Pseudomonas fluorescens. Proceedings of the National Academy of Sciences of the United States of America **101**:8072-8077.

Meyer, J. R. and R. Kassen. 2007. The effects of competition and predation on diversification in a model adaptive radiation. Nature **446**:432-435.

Neu, T. R., G. D. W. Swerhone, and J. R. Lawrence. 2001. Assessment of lectin-binding analysis for in situ detection of glycoconjugates in biofilm systems. Microbiology-Sgm **147**:299-313.

Newton, R. J., S. E. Jones, A. Eiler, K. D. McMahon, and S. Bertilsson. 2011. A Guide to the Natural History of Freshwater Lake Bacteria. Microbiology and Molecular Biology Reviews **75**:14-49.

Pal, R., S. Bala, M. Dadhwal, M. Kumar, G. Dhingra, O. Prakash, S. R. Prabagaran, S. Shivaji, J. Cullum, C. Holliger, and R. Lal. 2005. Hexachlorocyclohexane-degrading bacterial strains Sphingomonas paucimobilis B90A, UT26 and Sp+, having similar lin genes, represent three distinct species, Sphingobium indicum sp. nov., Sphingobium japonicum sp. nov. and Sphingobium francense sp. nov., and reclassification of [SFiphingomonas] chungbukensis as Sphingobium chungbukense comb. nov. International Journal of Systematic and Evolutionary Microbiology **55**:1965-1972.

Pernthaler, J. 2005. Predation on prokaryotes in the water column and its ecological implications. Nature Reviews Microbiology **3**:537-546.

Pernthaler, J., E. Zollner, F. Warnecke, and K. Jurgens. 2004. Bloom of filamentous bacteria in a mesotrophic lake: Identity and potential controlling mechanism. Applied and Environmental Microbiology **70**:6272-6281.

Puentes-Teliez, P. E., M. A. Hansen, S. J. Sorensen, and J. D. van Elsas. 2013. Adaptation and Heterogeneity of Escherichia coli MC1000 Growing in Complex Environments. Applied and Environmental Microbiology **79**:1008-1017.

Ruiz-González, C., J. P. Niño-García, and P. A. del Giorgio. 2015. Terrestrial origin of bacterial communities in complex boreal freshwater networks. Ecology Letters **18**:1198-1206.

Salcher, M. M., J. Pernthaler, and T. Posch. 2011. Seasonal bloom dynamics and ecophysiology of the freshwater sister clade of SAR11 bacteria 'that rule the waves' (LD12). Isme Journal **5**:1242-1252.

Schoener, T. W. 2011. The Newest Synthesis: Understanding the Interplay of Evolutionary and Ecological Dynamics. Science **331**:426-429.

Schuster, M., A. C. Hawkins, C. S. Harwood, and E. P. Greenberg. 2004. The Pseudomonas aeruginosa RpoS regulon and its relationship to quorum sensing. Molecular Microbiology **51**:973-985.

Schut, F., E. J. De Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button. 1993. Isolation of typical marine bacteria by dilution culture growth maintenance and characteristics of isolates under laboratory conditions. Applied and Environmental Microbiology **59**:2150-2160.

Schut, F., J. C. Gottschal, and R. A. Prins. 1997. Isolation and characterization of the marine ultrabacterium *Sphingomonas* sp. strain RB2256. FEMS Microbiology Reviews **20**:363-369.

Šimek, K. and T. H. Chrzanowski. 1992. Direct and indirect evidence of size-selective grazing on pelagic bacteria by freshwater nanoflagellates. Applied and Environmental Microbiology **58**:3715-3720.

Simon, M., H. P. Grossart, B. Schweitzer, and H. Ploug. 2002. Microbial ecology of organic aggregates in aquatic ecosystems. Aquatic Microbial Ecology **28**:175-211.

Stocker, R., J. R. Seymour, A. Samadani, D. E. Hunt, and M. F. Polz. 2008. Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches. Proceedings of the National Academy of Sciences **105**:4209-4214.

Ward, H. B. and G. C. Whipple. 1918. Fresh-water biology. John Wiley & sons, Incorporated.

Yamamichi, M., T. Yoshida, and A. Sasaki. 2011. Comparing the effects of rapid evolution and phenotypic plasticity on predator-prey dynamics. The American Naturalist **178**:287-304.

Yang, Z. and F. X. Kong. 2012. Formation of large colonies: a defense mechanism of Microcystis aeruginosa under continuous grazing pressure by flagellate Ochromonas sp. Journal of Limnology **71**:61-66.

Ying, B. W., T. Honda, S. Tsuru, S. Seno, H. Matsuda, Y. Kazuta, and T. Yomo. 2015. Evolutionary Consequence of a Trade-Off between Growth and Maintenance along with Ribosomal Damages. Plos One **10**.

Zotina, T., O. Köster, and F. Jüttner. 2003. Photoheterotrophy and light-dependent uptake of organic and organic nitrogenous compounds by Planktothrix rubescens under low irradiance. Freshwater Biology **48**:1859-1872.

Figures

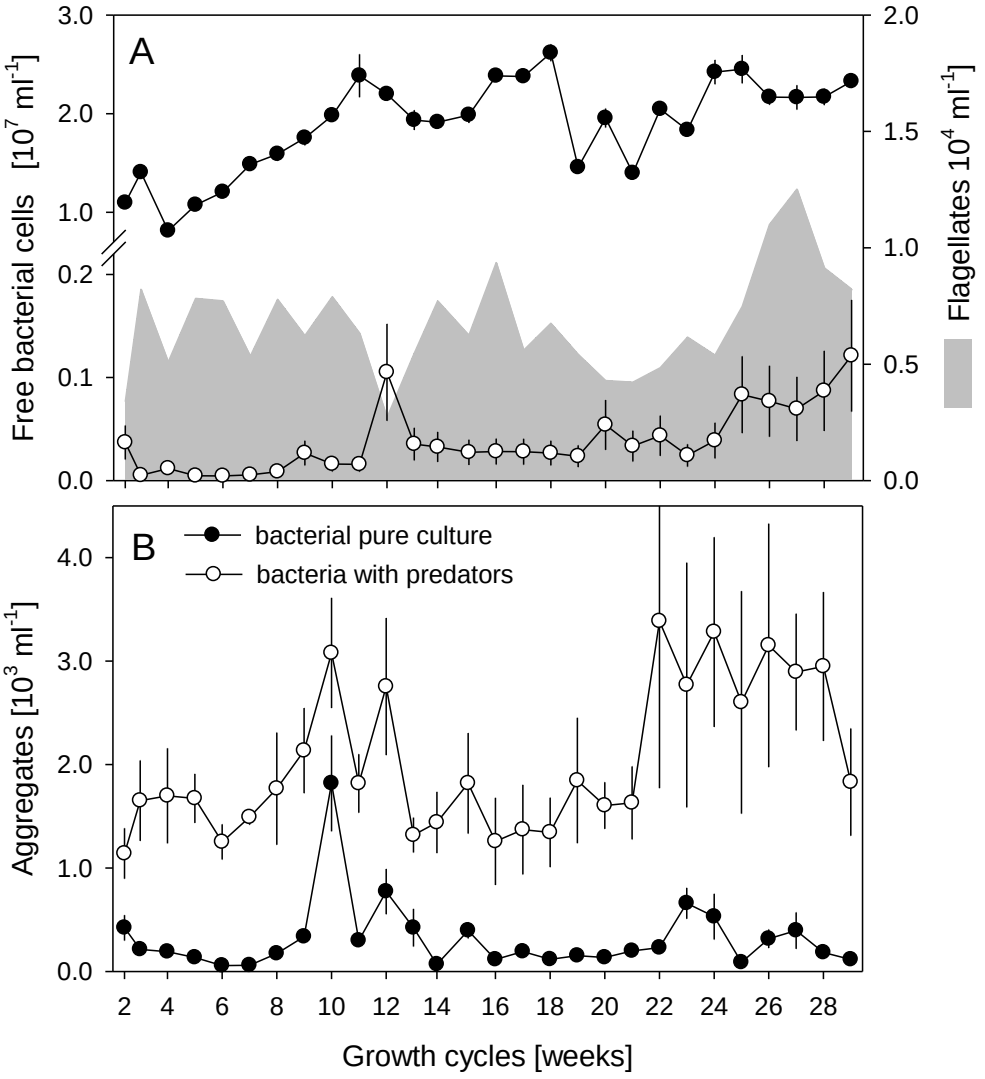

Fig. 1: Numbers of (A) free cells and (B) aggregates of *Sphingobium* sp. strain Z007 after 168 h of growth during sequential batch cultivation in 1:1000 diluted substrate concentration either in pure culture or together with the nanoflagellate *Poterioochromonas* sp. strain DS. The numbers of flagellates are depicted as grey area in the upper panel.

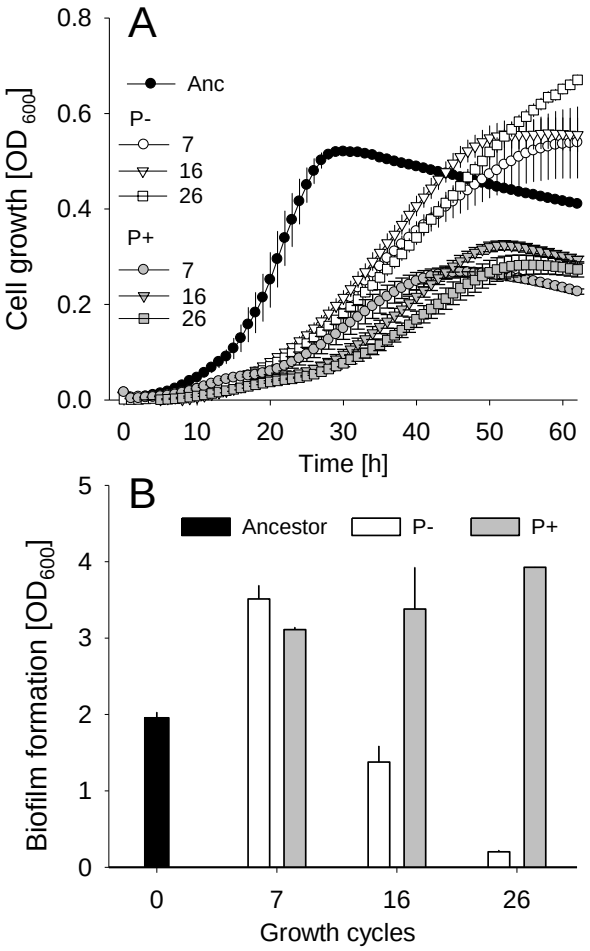

Fig. 2: (A) Growth curves on rich medium of the ancestor strain Sphingobium sp. strain Z007 and strains that evolved either without (P-; left) or with predation (P+; right) for 7, 16, and 26 growth cycles. Error bars are 1 standard deviation of either 3 biological replicates (ancestor) or of 3 isolates from 3 biological replicates per treatment (evolved strains). (B) Biofilm formation of the ancestor after 72 h of growth in rich medium and of strains isolated at different time points from the evolution experiment, as determined by crystal violet staining.

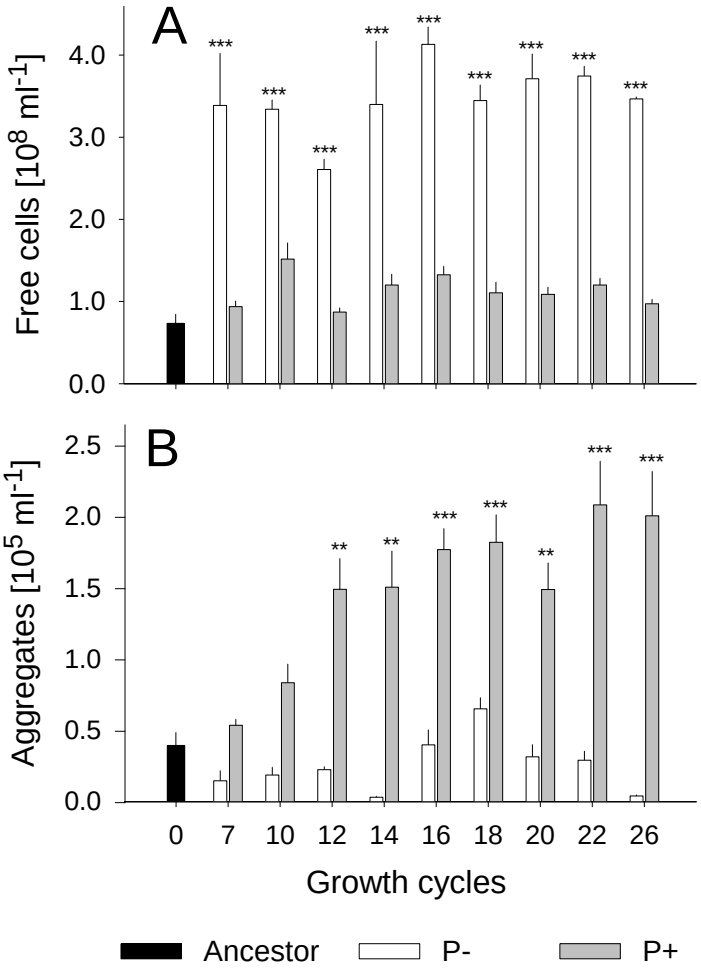

Fig. 3: Free cells (A) and aggregates (B) of evolved bacterial strains analysed after 96 h of growth (Fig. 2). The strains were isolated at different time points (growth cycles) from the different treatments of the evolution experiment. Error bars are 1 standard deviation (n=9). ** P< 0.01; *** P< 0.001.

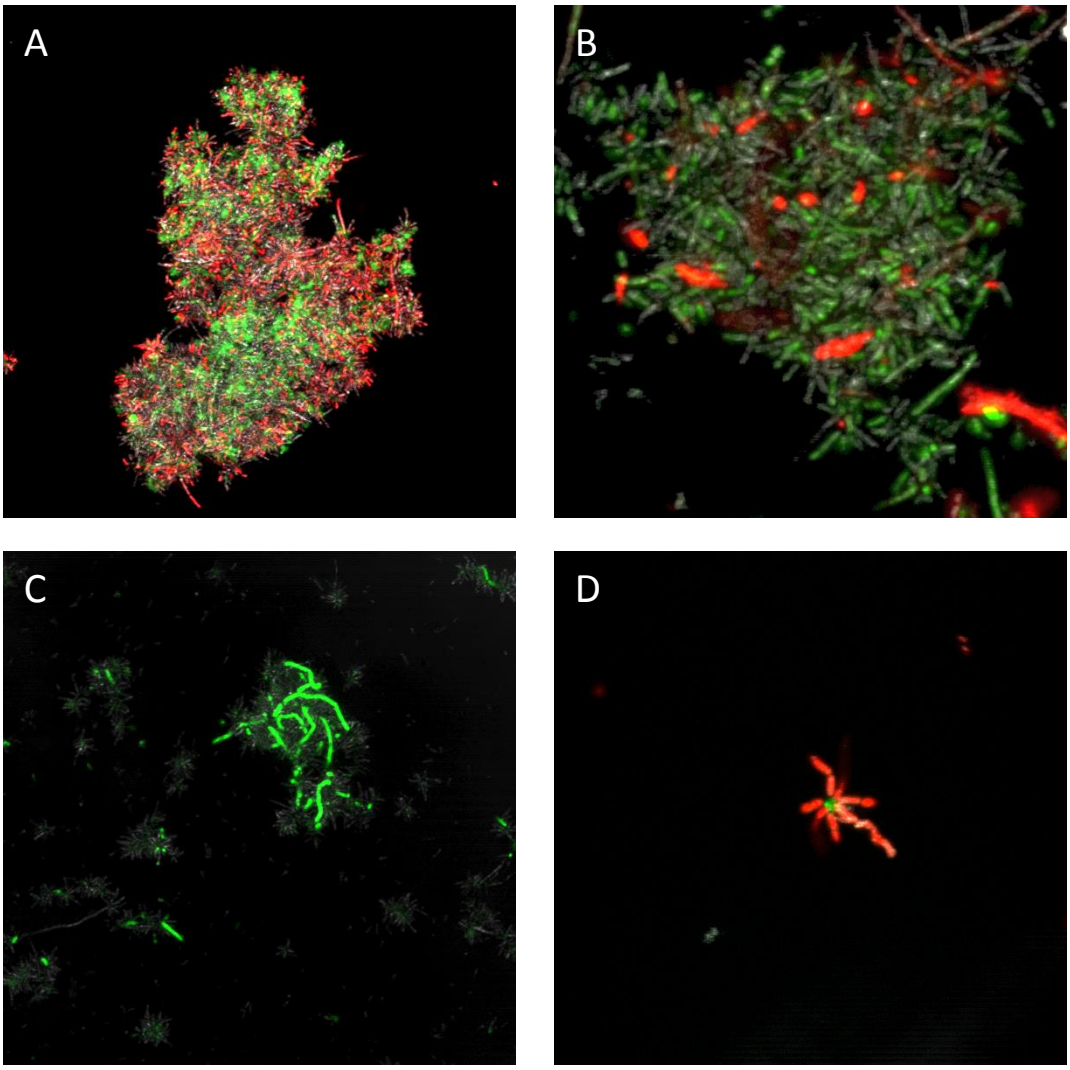

Fig. 4: Micrographs of aggregates of an evolved isolate (P+ treatment, 16 growth cycles) after 120 h of growth in rich medium. (A) Matrix stained with the lectin AAL (green) and cells stained with Syto60 (red). (B) Matrix elements stained with the lectin Lotus (red) and cells with Syto9 (green). (C) Filamentous cells stained with lectin WGA (green). (D) Rosette shaped aggregate or microcolony; the central area is stained with lectin HPA (green) and cells with Syto60 (red).

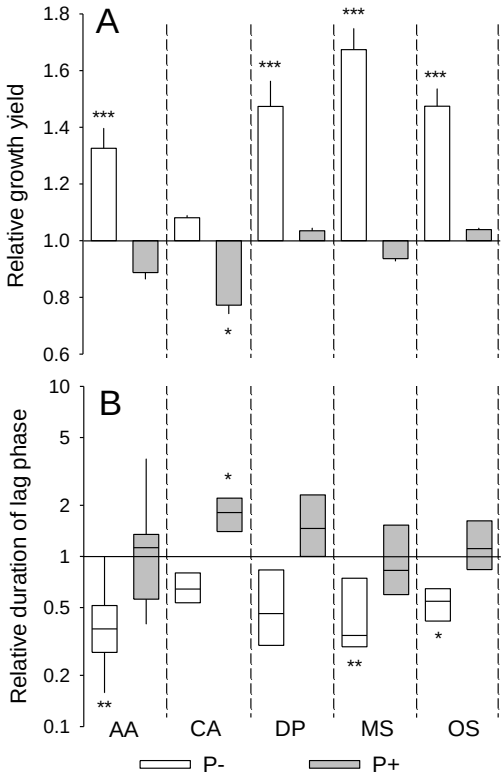

Fig. 5: Relative (A) growth yield or (B) duration of the lag phase (normalized to the ancestor) of evolved strains isolated after 26 growth cycles without (P-) or with (P+) predators on different substrate classes. * P<0.05; ** P< 0.01

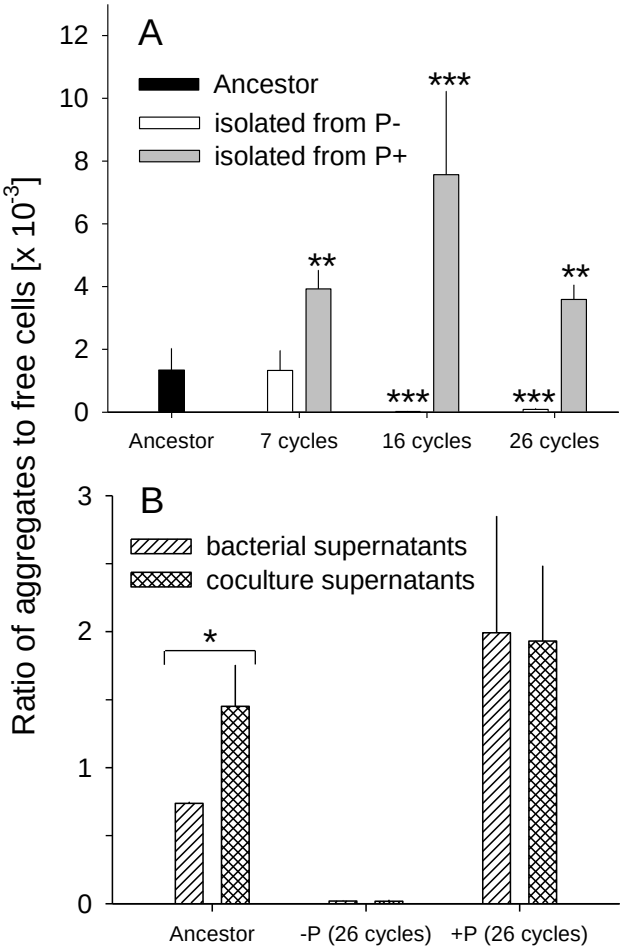

Fig. 6: Ratio of aggregates to free cells in the ancestor and in evolved strains isolated at different time points from treatments with (P+) and without (P-) predators. (A) Stains were grown in direct contact with *Poterioochromonas* sp. strain DS. (B) Strains were grown on supernatants of either a pure culture of the ancestor strain, or on supernatants of a co-culture of the ancestor and the flagellates. * P<0.05; ** P< 0.01;*** P< 0.001

Table 1. Lectins that bound to the matrix or surface structures of a *Sphingobium* sp. Z007 strain evolved with flagellates for 16 growth cycles

Lectin	Origin	Sugar specificity
AAL	*Aleuria aurantia*	Fucose linked to N-acetylglucosamine or N-acetyllactosamine
Lotus	*Tetragonolobus purpurea*	α linked L-fucose containing oligosaccharides
WGA	*Triticum vulgaris*	N-acetylglucosamine, sialic acid
PHA-E	*Phaseolus vulgaris*	Terminal galactose, N-acetylglucosamine and mannose residues
HPA	*Helix pomatia*	α-N-acetylgalactosamine
VVA	*Vicia villosa*	N-acetyl-D-galactosamine

Supplementary Information

Table S2: Consumption patterns of single carbon sources by the Sphingobium sp. strain Z007 ancestor, and by 3 isolates from replicate vessels evolved without (EvoA, B, C) or with (EvoPA, PB, PC) the bacterivorous flagellate *Poterioochromonas* sp. strain DS. The ancestor was assessed in triplicate, and the evolved strain in duplicates. Shaded in grey are substrates that were consumed by all strains and were, therefore, used in the subsequent growth assays.

Substrate	Ancestor	EvoA	EvoB	EvoC	EvoPA	EvoPB	EvoPC
Amino acids							
D-Alanine	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
L-Alanine	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
L-Glutamic Acid	+/-/+	+/+	+/+	+/+	+/+	+/+	+/+
N-Acetyl-L-Glutamic Acid	+/+/+	+/+	+/+	+/+	+/+	-/+	+/+
L-Glutamine	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
L-Isoleucine	+/+/+	+/+	+/+	+/+	-/+	+/+	+/+
L-Leucine	+/+/+	+/+	+/+	+/+	-/+	-/+	-/+
L-Proline	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Carboxylic acids							
4-Hydroxy Benzoic Acid	+/+/-	+/+	-/+	+/+	-/-	-/-	-/-
β-Hydroxy Butyric Acid	+/+/-	+/+	+/+	+/+	+/-	+/+	+/-
a-Keto-Valeric Acid	-/-/-	+/+	-/+	+/+	-/-	-/-	-/-
Acetic Acid	+/+/+	+/+	+/+	+/+	+/+	-/+	+/+
Citric Acid	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Formic Acid	+/+/+	+/+	+/+	+/+	-/-	+/+	+/-
Fumaric Acid	+/-/+	+/+	+/-	-/-	+/-	+/-	-/+
D-Gluconic Acid	+/-/-	+/+	+/-	-/-	+/-	+/-	-/-
L-Lactic Acid	+/-/+	-/-	-/-	-/-	-/-	-/-	-/-
D,L-Malic Acid	+/-/+	-/+	-/-	-/-	-/-	+/-	-/-
L-Malic Acid	+/-/+	-/+	+/+	-/+	+/+	+/+	+/+
Methyl Pyruvate	+/+/+	+/+	+/+	+/+	+/+	+/+	+/-
Propionic Acid	+/-/+	+/-	+/+	-/+	-/+	-/-	-/-
Pyruvic Acid	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Quinic Acid	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Succinic Acid	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+

Dipeptides							
L-Alanyl-Glycine	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Glycyl-L-Glutamic Acid	+/+/+	+/+	+/+	+/+	+/+	-/+	+/+
Glycyl-L-Proline	-/-/+	-/+	-/+	+/+	-/+	-/+	+/+
Monosaccharides							
L-Arabinose	+/+/+	+/+	+/+	+/+	-/+	+/+	+/+
D-Fucose	-/-/-	+/+	+/+	+/+	-/-	-/-	-/-
L-Fucose	+/-/+	+/+	+/+	+/+	+/+	+/+	-/+
D-Galactose	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
α-D_Glucose	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
L-Lyxose	-/-/-	+/+	+/+	+/+	-/-	-/-	-/-
D-Mannose	-/-/+	+/+	+/-	+/-	-/-	-/-	-/-
L-Rhamnose	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
D-Xylose	+/+/-	+/-	+/+	+/+	+/+	+/+	+/+
Oligosaccharides							
D-Cellobiose	+/+/+	+/+	+/+	+/+	+/+	+/+	-/+
Dextrin	-/-/+	+/+	+/+	+/+	-/+	-/+	-/+
Maltose	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Maltotriose	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Sucrose	+/-/+	+/+	+/+	+/+	+/+	+/+	+/+
D-Trehalose	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+
Polysaccharides							
α-Cyclodextrin	+/+/+	+/+	-/+	+/+	-/+	-/+	-/-
β-Cyclodextrin	+/+/+	+/+	+/+	+/+	+/+	-/+	+/+
Mannan	-/-/-	+/-	+/-	-/-	-/+	-/+	-/+
Pectin	+/+/+	+/+	+/+	+/+	+/+	+/+	-/+
Amid							
L-Alaninamide	-/-/-	+/+	+/+	-/+	-/+	-/-	-/-
Others							
Gelatin	+/+/+	+/+	+/+	+/+	+/+	+/+	+/+

4 Manuscript 2: Comparative genome analysis

4.1 Introduction to Manuscript 2

After we had exploited all possibilities to describe the phenotypes of the evolved strains, we decided to sequence genomes of the ancestor and evolved strains to gain further insight of how the different combinations of selection pressure would affect the respective genotypes.

The ancestor genome of *Sphingobium* sp. Z007 was sequenced as a reference using a combination of the PacBio and Illumina platforms; it was assembled with the h2gap for the PacBio reads and subsequently refined with the Illumina reads in CLC genomics Workbench. Two chromosomes and five plasmids were completely assembled, yielding a final genome size of 4.9 Mbp and an average GC content of 61.7%. These genomic features are similar to other so far sequenced sphingomonads (Aylward et al. 2013). Annotation revealed that all the essential genes to maintain the minimal metabolism of the cell (Gil et al. 2004) were found on the larger of the two chromosomes. Previous studies have suggested that having two chromosomes might be a risk spreading strategy of many bacterial species, dividing the chromosomes into a stable and flexible one (Cooper et al. 2010). In this concept, the larger chromosome usually carries genes that are important for essential processes in the cell and rearrangements in the genomic content could therefore have a severe impact on its fitness. The smaller chromosome is usually more flexible in its content and gives an adaptive advantage to certain habitats by carrying genes for specific metabolic pathways (Cooper et al. 2010). In contrast to chromosome 1 of *Sphingobium* sp. Z007 chromosome 2 does not possess a single unique essential gene. Moreover, a whole pathway for degradation of aromatic compounds was found exclusively on the smaller chromosome, which supports the notion that this chromosome is particularly important for the adaptation to specific environments. *Sphingobium* sp. Z007 also carries a conspicuously high number of mobile elements, a considerable proportion of which are located on chromosome 2. This indicates that the genome organization of chromosome 2 is more relaxed and that this chromosome experiences a lower extent of purifying selection than chromosome 1.

The comparison of the ancestor and evolved strains revealed an excision on chromosome 2 in all strains cultivated with the predator. The excised fragment appeared to be a remnant integrated plasmid, as well as a hot spot for the insertion of mobile elements, as it harboured nearly 80% of all transposable elements of chromosome 2. Since transposon integration is usually associated with gene loss (Cooper et al. 2001), the conspicuous accumulation of these detrimental mobile elements indicate that the former plasmid already was in a process of decay. However, the underlying molecular mechanism of the excision process is not clear. Homologous regions on both flanking sites suggest that the excision happened via homologous recombination. This type of recombination is an established method in molecular biology to genetically modify organisms (Thomason et al. 2001) and has been found to occur spontaneously in other microorganisms (Ochman et al. 2000).

Previous studies have shown that genome size reduction in bacteria is favoured in constant environments (Moran 2002) and comes together with fitness advantages within the constraint habitats they live in (Lee and Marx 2012). The excision had also a strong impact on the fitness of the strain evolved with predator. The immediate effect was that strains grew to a higher cell density after the excision than before, irrespective of predator presence. Furthermore, the ancestor and the strain evolved without predator were outgrown by the strains evolved with predator in direct competition experiments (again, with and without predator presence). The strains evolved with predators thus overcame the apparent growth disadvantage represented by aggregate formation and increased their competitive fitness. One strategy to surmount the trade-off between forming grazing protected cells and maximizing growth yield was presented by Thingstad et al. (Thingstad et al. 2005). The authors suggested that bacteria can avoid predation by using abundant (non-limiting) substrates to inflate cell volume beyond the limits of size-selectively feeding flagellates without allocating any limiting substrates to this process. We could show experimentally that a fitness gain was reached in our model organism despite high investment into aggregate formation. However, in contrast to the physiological mechanism suggested by Thingstad the *Sphingobium* sp. Z007 apparently overcame the trade-off by means of genome reduction.

The great impact on fitness caused by the excision would actually imply that it should have been a favourable strategy in all evolved strains. One explanation why

genome streamlining occurred only in strains evolved with predator is that it might be favourable for cells reproducing in aggregates and therefore was strongly selected against in strains evolved without predator (i.e., that largely gave up aggregation). Alternatively, it is possible that particular genes on the excision provided an advantage for the planktonic life-style and were therefore under positive selection.

So far, we have discussed only the advantages of genome streamlining. But deletions of large pieces of DNA bears also the risk of deleting potentially useful genes. The excised element showed an already advanced state of degradation as suggested by the high load of insertion sequences. However, between the mobile elements we found a fully functional metabolic pathway for the degradation of aromatic compounds, such as benzoate. As a consequence of the excision, this pathway was lost. Benzoate is a by-product of the plant metabolism and is often found in terrestrial environments. Furthermore, it was used in food industry as an antimicrobial agent (WHO 2000). The ancestor strain *Sphingobium* sp. Z007 can use benzoate as an additional growth substrate whereas growth of strains with the excision was inhibited in the presence of benzoate. As every coin has two sides, genome streamlining in our case narrowed down the possible niche space of the evolved strains.

Single nucleotide polymorphisms (SNPs) have been shown as drivers of evolution in laboratory strains and clinical isolates (Bryant et al. 2012). However, the evolved *Sphingobium* sp. Z007 strains had a conspicuously low number of SNPs (about 10) within the whole genome. In many cases it was impossible to assign SNPs a function because many of them were located within hypothetical genes and distributed all over the genome. Therefore, we still have no clear indication, which factors were ultimately responsible for the observed phenotypic variance (intensity of aggregate formation) between the strains with different evolutionary background.

A first hint to the reasons of the observed phenotypic differences between the two treatments was provided by the distribution of sequencing reads, which suggested a slight overexpression of plasmid 1 in the strains evolved under predation pressure. Among the genes encoded on this plasmid we found a complete quorum sensing and type 4 secretion system. Quorum sensing is involved in many collective processes, such as biofilm formation (Miller and Bassler 2001) and may also play a role in *Sphingobium* sp. Z007 to regulate processes that lead to the formation of aggregates. The type 4 secretion system was previously shown to be quorum sensing regulated (Rambow-Larsen et al. 2008) and the formed pili to establish contacts between cells can serve as a basic scaffold for a biofilm (Ghigo 2001) or aggregates (Bieber et al. 1998). Therefore, it is feasible that the type 4 secretion system played an important role in aggregate formation of *Sphingobium* sp. Z007. This hypothesis gets additional support from a previous finding: if we cultivated our bacteria under shaking conditions, cells were not able to form aggregates at all, likely because shearing forces for the formation of cell to cell contacts might be too high (Fig.6).

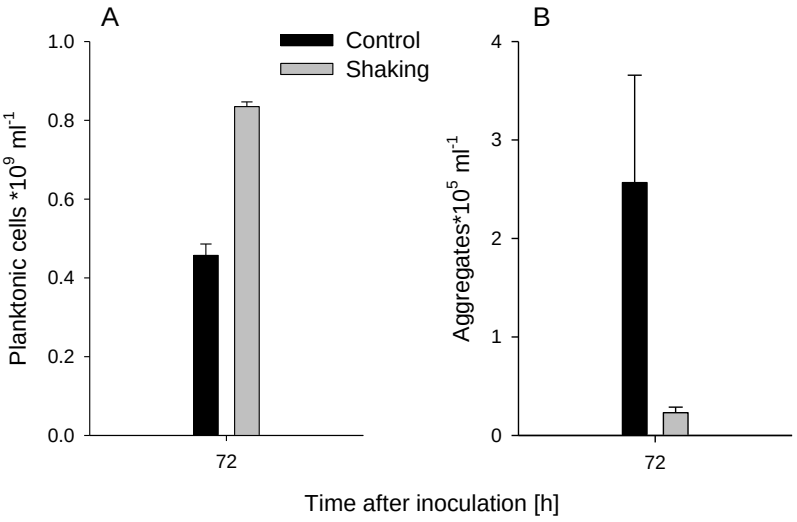

Fig 6: Growth of *Sphingobium* sp. Z007 free cells (A) and aggregates (B) during cultivation without disturbance and on a rotation shaker.

4.2 Manuscript 2

Letting go: genome streamlining allows for simultaneous adaptation to top-down and bottom-up constraints in a phenotypically plastic bacterial strain

Letting go: genome streamlining allows for simultaneous adaptation to top-down and bottom-up constraints in a phenotypically plastic bacterial strain

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Abstract

Resource limitation and predation mortality are major determinants of population dynamics, but optimization for either aspect is considered to imply a trade-off with respect to the other. For example, planktonic bacteria can form cell aggregates as defense against flagellate grazers, but this phenotype conveys a growth disadvantage due to diffusion limitation and high intraspecific competition. Here we present an example of a concomitant evolutionary optimization to both, substrate limitation and predation in an aggregate-forming freshwater bacterial isolate, and we elucidate the underlying genomic mechanism. Bacteria were propagated in serial batch culture in a nutrient restricted environment either with or without a bacterivorous flagellate. Strains isolated after 26 growth cycles of the predator-prey co-cultures formed as much total biomass as the ancestor, albeit largely reallocated to cell aggregates. An identical, ca. 273 kb genome fragment, likely the remnant of an integrated plasmid, was lost in 3 strains that had independently evolved with predators. These strains had significantly higher growth yield than others that were isolated from the same treatment before the excision event. Moreover, the isolates with the deletion always outcompeted both, the ancestor and the strains evolved without predators. At the same time, genome reduction led to a growth disadvantage in the presence of benzoate due to the loss of the respective degradation pathway, suggesting that niche constriction might be the price for the bidirectional optimization.

Introduction

Baas-Becking's classic proposition about the ubiquitous dispersal but concomitant environmental filtering of microorganisms (1) implies that bacteria are incessantly transported between habitats or biomes. Such transitions are likely accompanied by a qualitative or quantitative change of selective factors, often more than one. For example, terrestrial microbes are continuously introduced into freshwaters (2), where they face both, reduced substrate availability (3) and high mortality due to free-living protistan predators (4). Adaptations to the former constraint include motility to track patchy substrates (5), or the investment in high-affinity substrate uptake systems (6). Resistance to grazing can be realized by a variety of mechanisms, including the formation of protist-inedible (filamentous) morphotypes or cell aggregates (7). These two strategies are widely believed to reflect an obligatory trade-off between high mortality rates and high defense costs and are, therefore, considered to be mutually exclusive (8). However, it has also been proposed that some bacteria may successfully overcome the dilemma of simultaneous 'bottom up' limitation and high predation risk, e.g., by using non-essential substrates to inflate cells beyond a size that is ingestible for protists (9). So far, this so-called 'Winnie-the-Pooh' strategy is, by and large, a conceptual model backed by a limited set of field observations. Here we show that, starting with inducible predation resistance, it is possible to experimentally evolve bacteria that adopt such a strategy (i.e., simultaneously improve in growth efficiency and predation resistance), and we elucidate an underlying genomic mechanism.

Materials and Methods

Evolution experiment

Sphingobium sp. Z007 (16S rRNA gene accession number: FN293045), originally isolated from Lake Zurich (10) was evolved for 200 days (approximately 600 generations; 29 growth cycles) in sequential batch culture in Artificial Lake Water (ALW) medium (11) containing 1 mg l^{-1} of glucose, yeast extract and soytone peptone (i.e., 0.1 % of the substrate concentrations on which the strain had been maintained before). Bacterial cultures were grown at 18°C in triplicates of 100 ml, either with (P+) or without (P-) the bacterivorous flagellate *Poterioochromonas* sp. DS (12) . Every 168 h the cultures were 1:10 diluted into fresh ALW medium. Strains from all parallel treatments were isolated biweekly on solid DSMZ7 medium and stored at -80° C.

DNA extraction, Genome sequencing, assembly and annotation

Altogether 6 bacterial strains were genome sequenced: the ancestor and 5 evolved strains isolated from different experimental replicates (3 from P+, 2 from P-) after 26 re-inoculation cycles. The ancestor strain was first sequenced on the Pacific Bioscience RS platform, and all 6 strains were sequenced with the Illumina MySeq platform. Library preparation was done with PacBio® Template Prep Kit and the truseq DNA PCR-free kit (Illumina), respectively. To produce a high quality reference genome of the ancestor strain, the corresponding Illumina reads served for error correction of the *de novo* assembly from the Pacific Bioscience sequencing using CLC Genomics Workbench 7.0 (CLC bio). Gene calling and annotation of the reference genome was then performed with the RAST automated annotation pipeline (13, 14) and refined with the Artemis genome browser (15). In a second step, Illumina reads from the other strains were mapped to the ancestor reference genome and analysed with CLC Genomics Workbench 7.0. Stand-alone versions of Blast (16) and Dotter (www.ubuntu.com) were used for an in-depth analysis of differences between ancestor and evolved strains. Genomes of all sequenced isolates were submitted to European Nucleotide Archive under the accession no. ERS1026114 - ERS1026119.

Verification of excision

To confirm the excision in strains of the P+ treatments, 4 primers were designed that target its flanking regions (Supplement Table S1). DNA of the ancestor and evolved strains grown for 72h in DSMZ7 medium was extracted with the bacterial genome extraction Gen elute Kit (Sigma). Three separate PCR reactions were performed for each strain (see supplement figure S1 A+B for details): two with the flanking primers for each side of the excision to verify its occurrence and one with the forward primer of the left side with the reverse primer of the right side as an indicator of its absence.

Growth experiments on substrate-restricted media

Strains isolated from three replicates of the P+ treatment before and after the excision (isolated at 16 and 26 growth cycles) were inoculated directly from frozen stock cultures into 50 ml of DSMZ7 medium. After 72h the strains were transferred into ALW medium and grown for another 24h. Thereafter, $5x10^5$ bacteria ml^{-1} were inoculated into 50 ml of ALW medium either without flagellate predator or with $8x10^3$ *Poterioochromonas* sp. DS cells ml^{-1}. Bacterial cell densities were determined after 96h of growth in the dark at 18°C.

The ancestor and bacterial strains isolated after 26 growth cycles from each replicate of the evolution experiment were first pre-cultivated from frozen stock cultures as described above. $5x10^5$ bacteria ml^{-1} of each strain was then inoculated into triplicate 100 ml vessels with

50 ml ALW medium and grown in the dark at 18°C either with or without benzoate (1 g l^{-1}, Sigma). Cell densities were determined every 24 h for 72 h.

Competition assays on rich media

The ancestor and three strains isolated from separate vessels of the P+ and P- treatments after 26 growth cycles were pre-grown in DSMZ 7 medium for 72h as described above. Thereafter, strains were grown in DSMZ 7 medium at an initial bacterial cell density of $2{,}5x10^5$ cells ml^{-1}, either with or without 10^4 predator cells ml^{-1}. In the competition assays each strain from P+ was either grown in triplicates with the ancestor or fully factorial with each strain from P- (n=9). Quantification of cell densities and DNA extraction (Bacterial DNA extraction kit, Sigma) was carried out at the beginning of the experiment, and after 48h and 72h.

The cell ratios of competing strains was determined by qPCR. Primers for the 16s rRNA region of *Sphingobium* sp. Z007 were designed to determine total cell numbers. To distinguish strains from different evolutionary background a second primer pair was constructed for a gene involved in the metabolism of aromatic compounds (nitriloacetate monoxygenase component A, ntaA). It is situated on the excision and is, therefore, absent in all P+ strains. The specificity of the primers was tested in pure cultures of the different strains. qPCR amplification was performed with the CFX96 real-time PCR detection system (Bio Rad). Copy numbers of genes (corrected by copy numbers per cell) were used to determine frequencies of strains at the beginning and the end of the experiment. Changes in relative frequencies between the start and the end of the experiments was used to calculate relative fitness (W_r) according the equation: $W_r = (a_t(1-a_0)/(a_0(1-a_t))$, where a_0 and a_t are the cell densities of the focal strain a at the beginning and the end, respectively (17).

Cell numbers, biomass

Bacterial cell densities were determined by flow cytometry. Subsamples of 1 ml were fixed with glutaraldehyde (v/v 2.5%) and stained for 20 min with 4',6-diamidino-2-phenylindole (DAPI, 1 µg ml^{-1}). Cell numbers were measured with an Influx V-Gs cell sorter (Becton Dickinson Inc.). DAPI fluorescence was excited with a UV laser (60 mW, 355 nm), and a blue laser (200 mW, 488 nm) was used for side scatter light (SSC) and the autofluorescence of *Poterioochromonas* sp. DS. Bacterial cells were detected SSC versus DAPI fluorescence (431 nm) and flagellates were determined using SSC versus green fluorescence (531 nm).

The total protein biomass of the ancestor strain and of bacteria isolated after 26 re-inoculation cycles from P+ and P- was determined in pre-grown cultures after 72 h on DSMZ 7 medium. Subsamples were concentrated by centrifugation, the pellet was resuspended in inorganic ALW, and cells were disrupted by 4x 30 s of sonication (Skan, Sonifier 250). Protein fluorescence was measured on a Qubit 2.0 (Invitrogen) after staining with Qubit protein reagent

according to the manufacturer's specification. The biomass of free cells was determined separately by pre-filtration of subsamples through 3 µm pore size filters.

Results

Adaptation to substrate limitation and predation

Compared to the ancestor, cultures evolving without predation showed a gradual but moderate increase in final cell densities during the experiment (Fig. 1a). The cell yield of the P- cultures after 26 growth cycles was on average 1.7 times higher than the beginning. The P+ cultures showed a more drastic adaptation, and their final cell densities were 15 times higher than their initial numbers. Strains isolated from the P+ treatment after 26 growth cycles that were grown in rich DSMZ 7 medium formed slightly (but not significantly) higher total biomass than the other strains (Fig. 1b). In the P- strains, a significantly larger fraction of total biomass was present in free cells, in agreement with the notion of aggregate formation being a predation defence.

Genomic organization and characteristics of the ancestor

The genome of the *Sphingobium* sp. Z007 ancestor strain consists of two circular chromosomes and five plasmids with a final genome size of 4.9 Mbp. 3276 from a total of 4998 coding sequences (CDS) could be functionally annotated. A summary for each genetic element is listed in Table 1. The genome includes 4 rRNA operons and 60 tRNA genes that cover all amino acids. The closest relative of *Sphingobium* sp. Z007 is *Sphingobium japonicum,* with a 16s rRNA gene identity of 98%. Chromosome 1 contains all genes essential to maintain a living cell (18). Chromosome 2 is only half the size of chromosome 1; it mainly harbours genes involved in substrate degradation, as well as nearly half of all transposons. We also found genes involved in quorum sensing, namely one autoinducer gene on plasmid 1 and three receptor genes on chromosomes 1, 2 and plasmid 1.

Loss of genome fragment

All 3 strains from different experimental replicates of the P+ treatment had a smaller genome size than both, the ancestor and the P- strains. The lost genomic fragment is located on chromosome 2 (Fig. 2); it is nearly 274 kb in size and contains 306 CDS, 164 of which could be sorted into different metabolic groups. The most abundant annotated CDS on the excision are mobile elements (insertion sequences from different families) and unique sets of genes involved in the metabolism of aromatic compounds. Consequently, the P+ strains have completely lost these degradation pathways. Mobile elements and large stretches of repeats are found on both flanking sites of the excision and might in fact be responsible for its loss. Screening of a collection of >50 isolates from different replicates and time points of the P+

treatment by the specific PCR assay (Suppl. Fig. 1) revealed that the excision had occurred after 20, 22, and 24 re-inoculation cycles in the three isolates, respectively (corresponding to 136, 150, and 164 days).

Growth efficiency after genome streamlining

The growth of P+ strains from before (16 growth cycles) and after (26 growth cycles) the excision on oligotrophic medium revealed beneficial effects of genome streamlining. Significantly higher growth yield of all strains with reduced genomes was observed, and this was equally pronounced in the presence and absence of predators (Fig. 3). To further verify that change in growth was mainly related to the loss of the genomic element, a strain isolated two growth cycles before the excision (24 growth cycles) was also tested. No significant differences in growth compared to its ancestor isolated after 16 re-inoculation cycles was observed (data not shown).

Competition between evolved strains

To examine the competitiveness between the evolved strains at substrate-rich conditions and between P+ strains and the ancestor we measured their fitness in co-culture (change in relative frequency of marker genes). Pre-experiments confirmed that comparable abundance estimates of the ancestor strain were obtained using primers either for 16s rRNA genes or for the excision region (data not shown). qPCR efficiencies, as determined from standard curves, were always between 98 and 102 %. Significantly higher fitness of the P+ than the P- strains was observed, both, in the absence (one-sample t-test, $P < 0.0001$, $n=22$) and the presence ($P < 0.0001$, $n =27$) of predators (Fig. 4, left panel). Similarly, there were always significant differences in fitness between P+ strains and the ancestor (Fig. 4, right panel; without predators: $P < 0.001$, $n = 9$; with predators: $P < 0.0001$, $n = 9$).

Growth in the presence of benzoate

One consequence of the genome reduction in P+ strains was the loss of genes involved in the metabolism of aromatic compounds, including the complete pathway for benzoate degradation (Fig. 3). The ancestor and the P- strains, both with an intact gene cluster for this metabolic function, grew to higher densities in media containing benzoate than in control treatments (Fig. 5), indicating that they could utilize this compound as an additional substrate source. By contrast benzoate negatively affected the growth of the P+ strains with reduced genomes.

Discussion

Sphingomonadaceae are distributed across a range of non-aquatic and aquatic habitats. Their genome evolution is characterized by a high extent of horizontal gene transfer, recombination and genome rearrangements (19). Genome reduction within this phylogenetic lineage appears to reflect ecological transitions to less substrate-rich environments (20), in agreement with a general trend of smaller genomes in bacteria from aquatic than from terrestrial habitats (21).

While the smallest, most eroded genomes are found in obligate endosymbionts (22, 23), the hallmark examples of extreme genome reduction in free-living bacteria are from oligotrophic marine and freshwater environments (24, 25). Genome streamlining of waterborne bacteria is considered to be a consequence of positive selection rather than of genetic drift (26), in order to increase growth efficiency in a resource-limited environment. In our experiments, all strains evolved towards increasing their growth yield at thousandfold reduced substrate levels (Fig. 1A). However, genome reduction was exclusively triggered at high predation mortality, and only after several 100 generations. In the absence of predators, growth efficiency could be increased by re-tuning the resource allocation trade-off between growth and defence (27), .i.e., by largely abandoning the aggregated phenotype (Fig. 1B). This evolutionary path was no option for the P+ strains, which maintained or even increase aggregate formation, likely due to the high mortality inflicted on the planktonic subpopulation.

During the 1-week growth cycles, flagellates removed approximately 80% of newly formed free bacteria cells already after 24 h and subsequently reduced them to <25% of their maximal abundances. Consequently, the starter populations of *Sphingobium* sp. Z007 at each re-inoculation in P+ were 2 to 3 orders of magnitude smaller than in P-. Evolutionary rates in small populations under severe selection pressure are typically limited by the extent of available variability (28), which may lead to a disproportional occurrence of mutator strains (29). Such a growth scenario might also favour the success of a single, more radical adaptation as represented by the genomic excision. Moreover, 10 to 100 times more cells in the freshly re-inoculated P+ variant originated from aggregates rather than from planktonic bacteria, so that this phenotype constituted by far the largest effective subpopulation. It is, thus, conceivable that the excision event occurred in the aggregated phenotype, and was rapidly established by conveying a growth advantage (Fig. 4) to cells that were not subjected to predation mortality. This might also explain why it was never observed in the P- strains. Alternatively, the excised genome fragment might have harboured elements that were beneficial for planktonic growth.

Metabolic costs associated with larger genome size arise from regulation overhead and to a lesser extent from actual synthesis (30). The superior growth of P+ strains after the excision on oligotrophic media irrespective of predation (Fig. 3) suggests that the overall fitness benefit of the genome reduction for the P+ strains may have exceeded the 5% of saved nucleotides. Interestingly, it also bestowed an advantage on P+ strains at the original substrate-rich growth conditions, and they were able to outcompete both, the ancestor and the P- strains, irrespective of predation (Fig. 4). Thus, these strains might be regarded as the first examples for an experimentally induced 'Winnie-the-Pooh' type of adaptation with simultaneously increased growth and defence related fitness aspects. However, the mechanism by which this was achieved is unrelated to the originally suggested one (9).

The excised genome region most likely represents an eroded remnant of an integrated plasmid, as deduced from plasmid-related genes, and the disproportionally high number of transposable elements. At the same time, it also harboured a fully functional pathway for the degradation of aromatic compounds (Fig. 2), as demonstrated by enhanced cell yield of the ancestor and P- strains in the presence of benzoate (Fig. 5). By contrast, this compound, -which is known for its antimicrobial activity, but is also common in terrestrial habitats such as the rhizosphere (31)- constituted a growth disadvantage for the P+ strains. Thus, the price of genome reduction in *Sphingobium* sp. strain Z007 seems to be a constriction of its potential niche space.

References:

1. Baas-Becking LGM (1934) *Geobiologie; of inleiding tot de milieukunde* (WP Van Stockum & Zoon NV).
2. Ruiz-González C, Niño-García JP, & del Giorgio PA (2015) Terrestrial origin of bacterial communities in complex boreal freshwater networks. *Ecology Letters* 18(11):1198-1206.
3. Guildford SJ & Hecky RE (2000) Total nitrogen, total phosphorus, and nutrient limitation in lakes and oceans: Is there a common relationship? *Limnology and Oceanography* 45(6):1213-1223.
4. Pernthaler J (2005) Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Micro* 3(7):537-546.
5. Mitchell JG & Kogure K (2006) Bacterial motility: links to the environment and a driving force for microbial physics. *FEMS Microbiology Ecology* 55(1):3-16.
6. Tang K, Jiao N, Liu K, Zhang Y, & Li S (2012) Distribution and Functions of TonB-Dependent Transporters in Marine Bacteria and Environments: Implications for Dissolved Organic Matter Utilization. *PLoS ONE* 7(7):e41204.
7. Corno G & Jürgens K (2008) Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. *Environmental Microbiology* 10(10):2857-2871.
8. Lenski RE & Levin BR (1985) Constraints on the coevolution of bacteria and virulent phage: a model, some experiments, and predictions for natural communities. *American Naturalist*:585-602.
9. Thingstad TF, Øvreås L, Egge JK, Løvdal T, & Heldal M (2005) Use of non-limiting substrates to increase size; a generic strategy to simultaneously optimize uptake and minimize predation in pelagic osmotrophs? *Ecology Letters* 8(7):675-682.
10. Blom JF, Zimmermann YS, Ammann T, & Pernthaler J (2010) Scent of Danger: Floc Formation by a Freshwater Bacterium Is Induced by Supernatants from a Predator-Prey Coculture. *Applied and Environmental Microbiology* 76(18):6156-6163.
11. Zotina T, Köster O, & Jüttner F (2003) Photoheterotrophy and light-dependent uptake of organic and organic nitrogenous compounds by Planktothrix rubescens under low irradiance. *Freshwater Biology* 48(10):1859-1872.
12. Hahn MW & Höfle MG (1999) Flagellate Predation on a Bacterial Model Community: Interplay of Size-Selective Grazing, Specific Bacterial Cell Size, and Bacterial Community Composition. *Applied and Environmental Microbiology* 65(11):4863-4872.
13. Aziz R*, et al.* (2008) The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics* 9(1):75.
14. Overbeek R*, et al.* (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research* 42(Database issue):D206-D214.
15. Rutherford K*, et al.* (2000) Artemis: sequence visualization and annotation. *Bioinformatics* 16(10):944-945.
16. Altschul SF, Gish W, Miller W, Myers EW, & Lipman DJ (1990) BASIC LOCAL ALIGNMENT SEARCH TOOL. *Journal of Molecular Biology* 215(3):403-410.
17. Adin Ross-Gillespie, Andy Gardner, Stuart A. West, & Ashleigh S. Griffin (2007) Frequency Dependence and Cooperation: Theory and a Test with Bacteria. *The American Naturalist* 170(3):331-342.
18. Gil R, Silva FJ, Peretó J, & Moya A (2004) Determination of the Core of a Minimal Bacterial Gene Set. *Microbiology and Molecular Biology Reviews* 68(3):518-537.
19. Aylward FO*, et al.* (2013) Comparison of 26 Sphingomonad Genomes Reveals Diverse Environmental Adaptations and Biodegradative Capabilities. *Applied and Environmental Microbiology* 79(12):3724-3733.
20. Lauro FM*, et al.* (2009) The genomic basis of trophic strategy in marine bacteria. *Proceedings of the National Academy of Sciences* 106(37):15527-15533.

21. Dini-Andreote F, Andreote F, Araújo W, Trevors J, & van Elsas J (2012) Bacterial Genomes: Habitat Specificity and Uncharted Organisms. (Translated from English) *Microb Ecol* 64(1):1-7 (in English).
22. Andersson SGE*, et al.* (1998) The genome sequence of Rickettsia prowazekii and the origin of mitochondria. *Nature* 396(6707):133-140.
23. Stephens RS*, et al.* (1998) Genome Sequence of an Obligate Intracellular Pathogen of Humans: Chlamydia trachomatis. *Science* 282(5389):754-759.
24. Giovannoni SJ*, et al.* (2005) Genome Streamlining in a Cosmopolitan Oceanic Bacterium. *Science* 309(5738):1242-1245.
25. Dufresne A*, et al.* (2003) Genome sequence of the cyanobacterium Prochlorococcus marinus SS120, a nearly minimal oxyphototrophic genome. *Proceedings of the National Academy of Sciences* 100(17):10020-10025.
26. Giovannoni SJ, Cameron Thrash J, & Temperton B (2014) Implications of streamlining theory for microbial ecology. *ISME J* 8(8):1553-1565.
27. Cressler C, King A, & Werner E (2010) Interactions between Behavioral and Life-History Trade-Offs in the Evolution of Integrated Predator-Defense Plasticity. *American Naturalist, The* 176(3):276-288.
28. Gillespie JH (1999) The Role of Population Size in Molecular Evolution. *Theoretical Population Biology* 55(2):145-156.
29. Handel A & Rozen DE (2009) The impact of population size on the evolution of asexual microbes on smooth versus rugged fitness landscapes. *BMC Evolutionary Biology* 9(1):1-10.
30. Ranea JAG, Grant A, Thornton JM, & Orengo CA (2005) Microeconomic principles explain an optimal genome size in bacteria. *Trends in Genetics* 21(1):21-25.
31. Whitehead DC, Dibb H, & Hartley RD (1981) Extractant ph and the release of phenolic compounds from soils, plant roots and leaf litter. *Soil Biology and Biochemistry* 13(5):343-348.
32. Muyzer G & Ramsing NB (1995) Molecular methods to study the organization of microbial communities. *Water Science and Technology* 32(8):1-9.

Figures

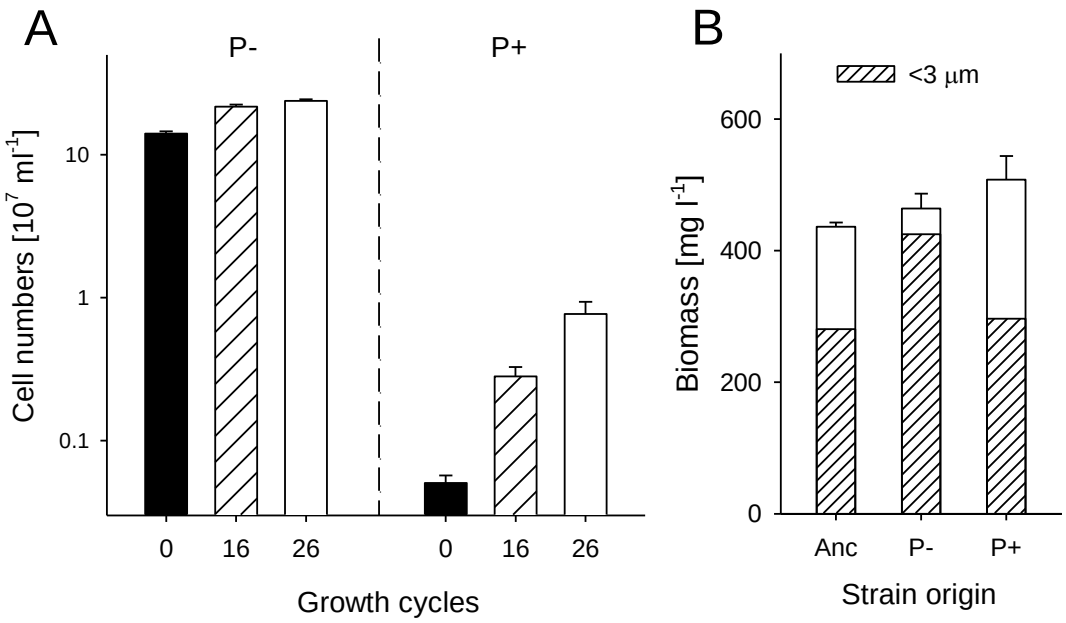

Fig. 1

Increase of total growth yield during experimental evolution and reallocation of biomass between planktonic and aggregate associated cells. (A) Cell densities of the ancestor *Sphingobium* sp. Z007 strain (168h of growth at oligotrophic conditions) and of 3 strains evolving without (P-) or with (P+) predators that were isolated after 16 and 26 growth cycles. (B) Biomass of planktonic cells (hatched) and aggregates (white) of the ancestor and of strains with different evolutionary background isolated after 26 growth cycles. Error bars: 1 standard deviation (ancestor: n=3; P- and P+: n=9).

Table 1: Genomic features of *Sphingobium* sp. Z007

Features of Sphingobium sp. Z007	CC1	CC2	P1	P2	P3	P4	P5
Size [bp]	3251406	1165775	298459	168066	30817	13179	10308
G+C (%)	63.9	62.4	62.4	61.9	63	59.1	59.5
Protein coding (bp)	2952693	1064189	266541	149157	26997	11028	8667
Protein coding (%)	90.8	91.2	89.3	88.7	90.7	83.7	84.1
Protein coding genes (no)	3240	1092	333	207	37	21	18
with functional assignment	2137	654	177	76	18	8	5
hypothetical genes	1103	438	156	131	19	13	13
Insertion sequences (no)	57	74	25	11	4	1	1
Ribosomal RNAs	1	3	-	-	-	-	-
Transfer RNAs	51	9	-	-	-	-	-

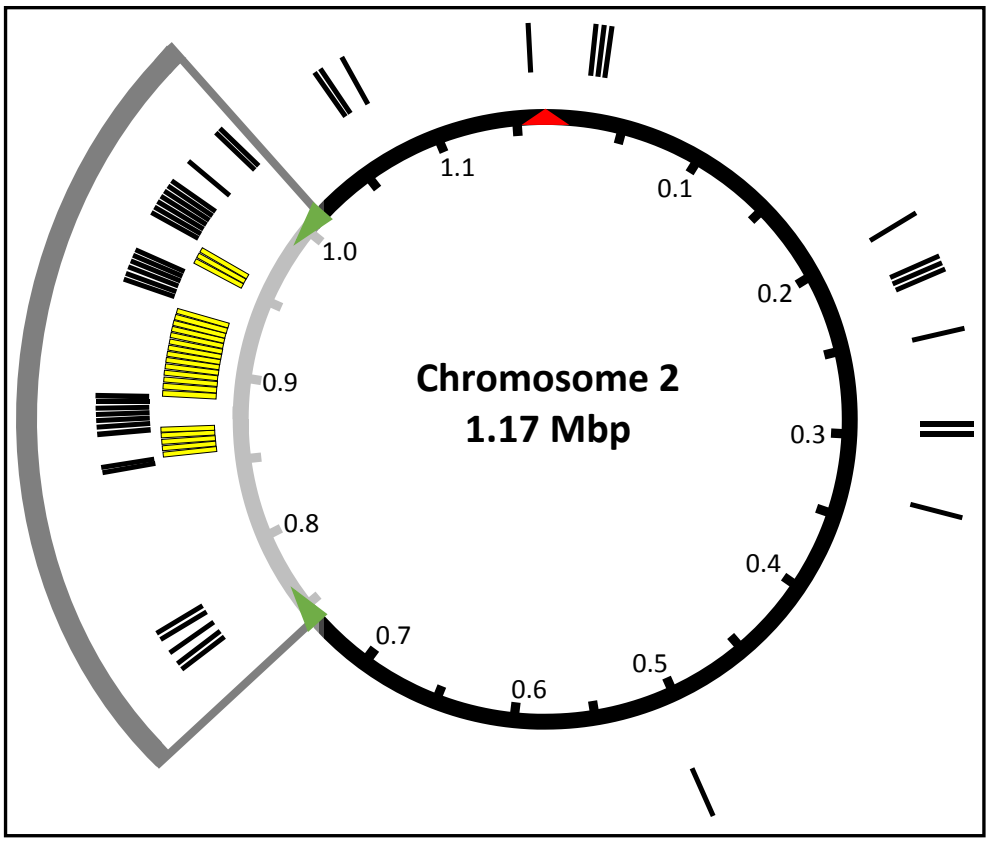

Fig. 2

Location of the genomic excision (marked in grey) on chromosome 2, and distribution of transposable elements (black bars) and genes involved in metabolism of aromatic compounds (yellow bars). The size of the excision was approximately 273kbp.

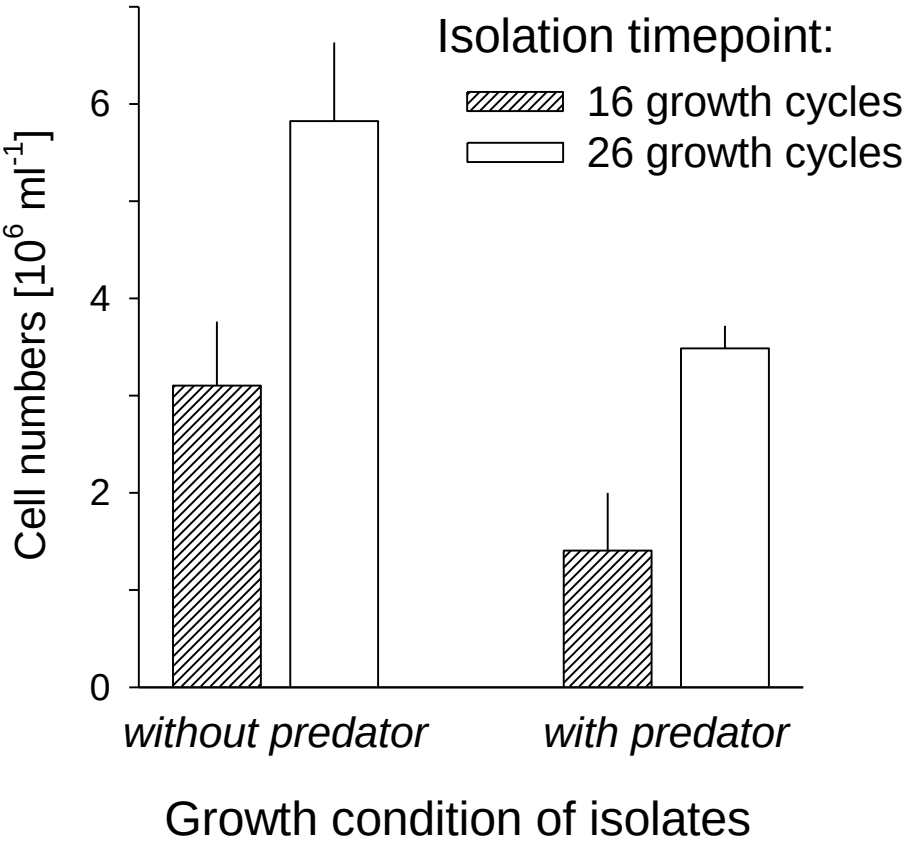

Fig. 3

Cell densities after 96 h of growth in nutrient limited medium of three independently evolved strains isolated before (16 growth cycles) and after (26 growth cycles) genome reduction. Strains were cultivated either without or with predator. The difference between strains were significant at $P < 0.001$ (Tukey's range test). Error bars: 1 standard deviation (n=18).

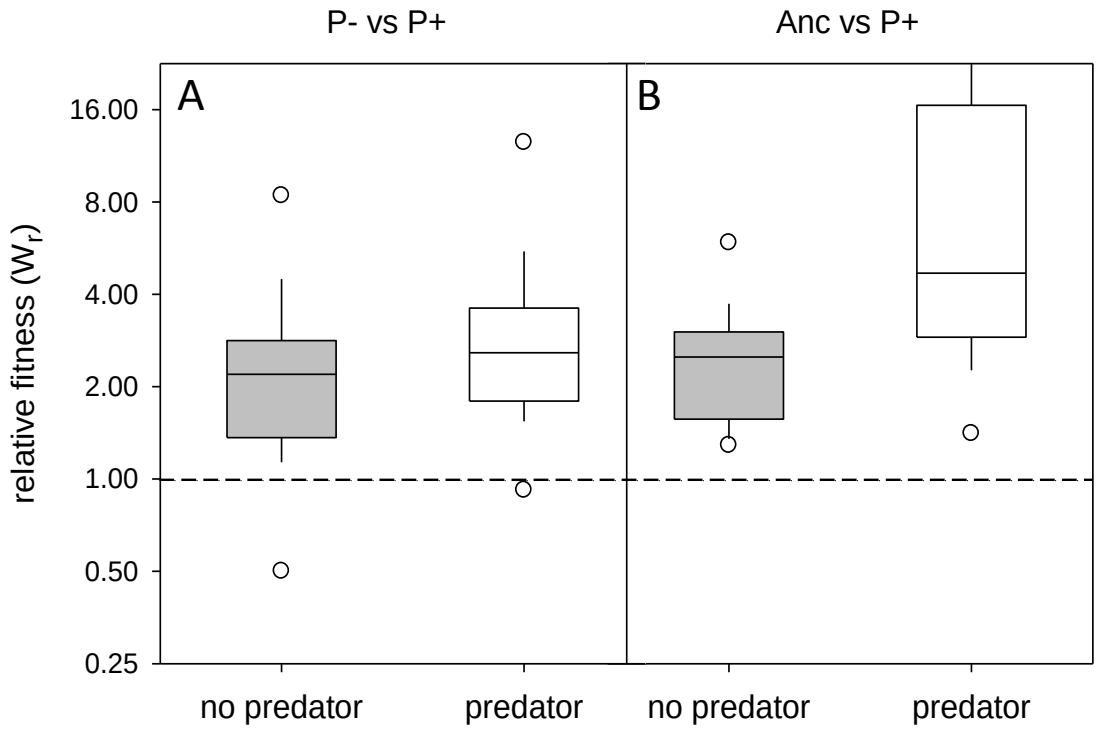

Fig. 4

Competitive performance of strains evolved with predators: Left panel: Differences in fitness between strains from the P+ and P- treatments (26 growth cycles) cultivated either without (n=22; P< 0.0001, one-sample t-test) or with predators (n=26; P< 0.0001). (B) Differences in fitness between strains from the P+ and the ancestor without (n=9; P< 0.001) or with predators (n=9; P< 0.0001)

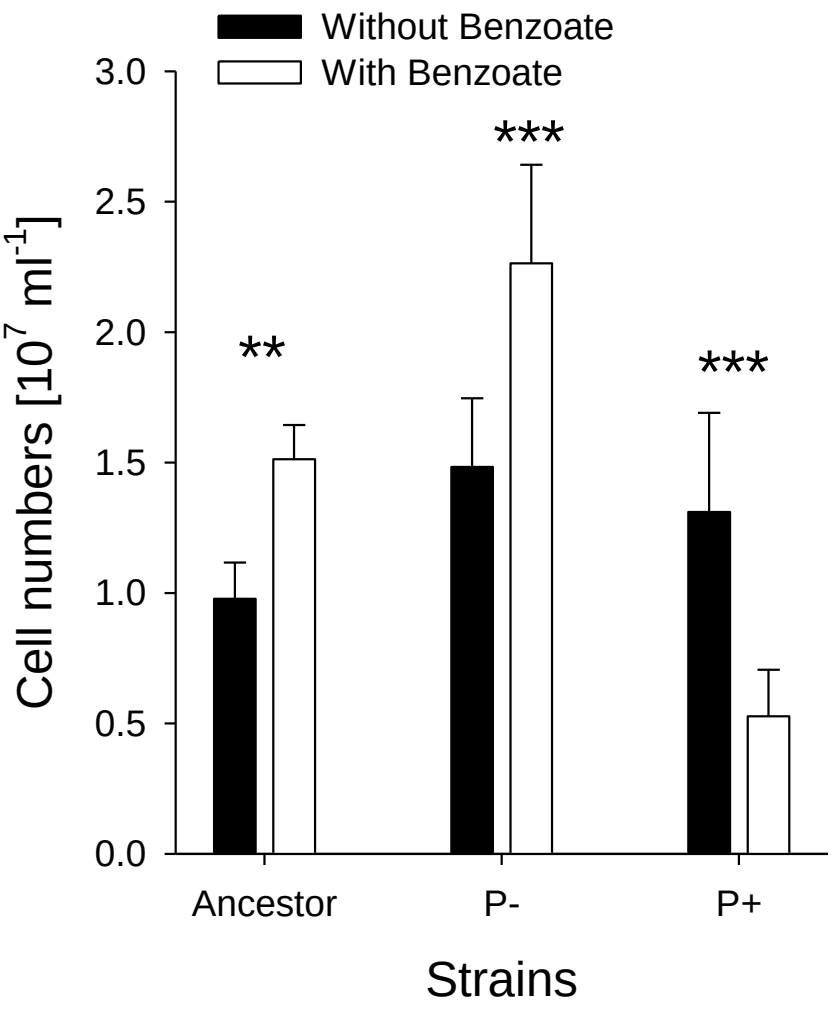

Fig. 5

Comparison of growth of the ancestor and of evolved strains (isolated after 26 growth cycles) in the presence or absence of benzoate. Difference between treatments were significant at $P < 0.001$ (Tukey's range test). Error bars: 1 standard deviation (ancestor, n=3, P+ and P-: n=9).

Supplementary

Table S1. Primers used for 16s rDNA sequencing, confirmation of excision and qPCR.

Name	5' → 3' forward primer	5' → 3' reverse primer
GM3f and GM4r (32)	AGAGTTTGATCMTGGC	TACCTTGTTACGACTT
c2_del_leftend	CGACGCTGTTCGATCACTC (A)	GGCCATTCGGACCTATGTCG (B)
c2_del_rightend	TACGCGGTCATACGGTCAGG (C)	GCGTCGAGTGGCCGAGAATG (D)
QPCR_16s	CAGCCTGGTCCATTATGACA	GTTGCCGAATGGTCAGATTC
QPCR_excision	GTGATAAGCCGGAGGAAGGT	CGCTCTCGAGTTGCAGAGAA

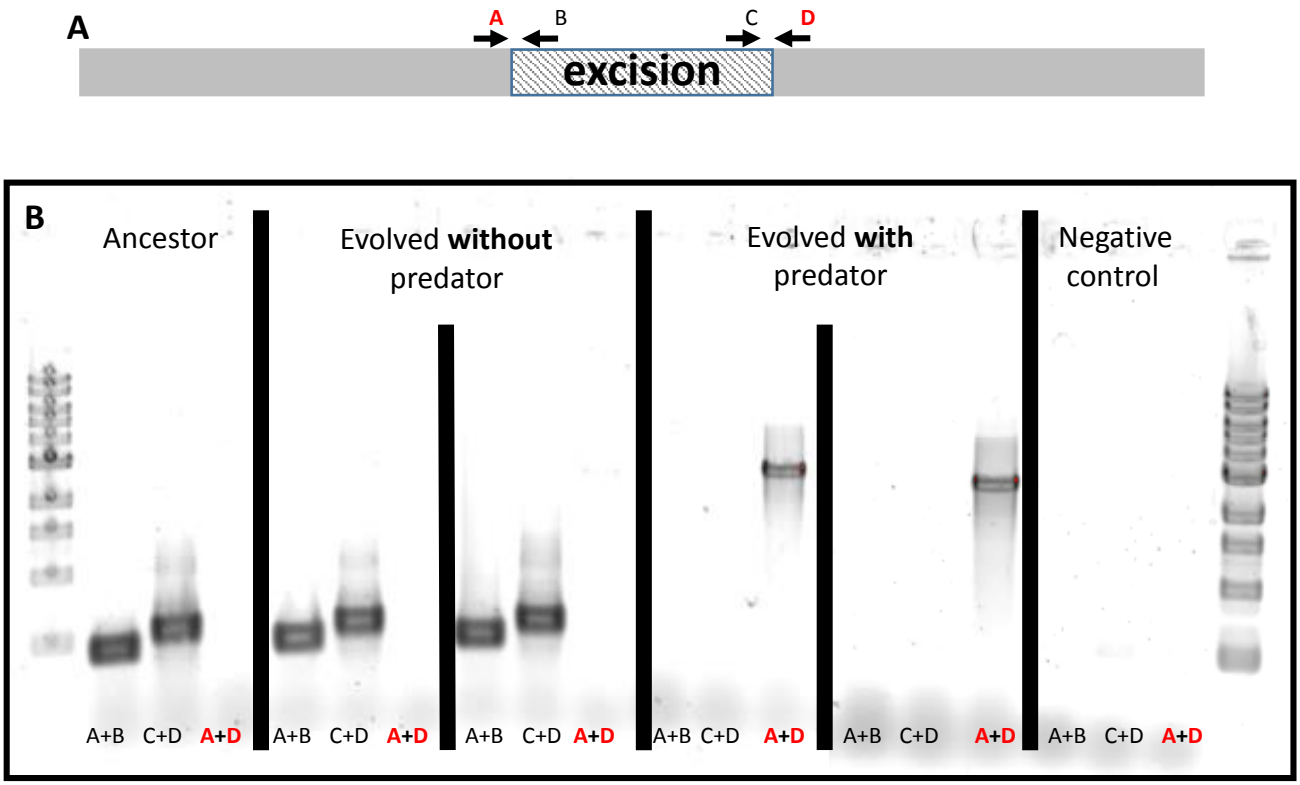

Fig. S1

PCR confirmation of excision. (A) Schematic drawing of primer binding sites on the flanking sites of the excision. (B) PCR product of flanking sites were only present in the Ancestor and strain evolved without predator whereas only the forward primer of the left flanking site in combination with the reverse primer of the right flanking site gave a product in the strains evolved with predation, which confirmed excision of that genomic region.

5 Manuscript 3: Alternating presence of predators

5.1 Introduction to Manuscript 3

The ability of *Sphingobium* sp. Z007 to sense the presence of predators is a very sensitive phenotype that is easily lost. Several propagations of this strain in nutrient rich medium result in a rapid decrease of inducible aggregate formation. Our long-term evolutionary experiment showed that the strains grown for 200 days either with or without predators had consistently lost their ability to induce aggregation in presence of a predator. It has been shown previously that constant environments may lead to a reduction of phenotypic complexity (Maughan and Nicholson 2011). A theoretical analysis illustrated that such plasticity is only favoured in unstable environments but immediately loses its advantage if conditions stabilize (Yamamichi et al. 2011). Based on our observation we wanted to test by an experimental evolution approach if an alternating regime of presence and absence of predators would maintain prey sensitivity and if such phenotypic plasticity is reduced upon constant presence or absence of a predator. For this, we employed an experimental evolution approach, where we used our previous experimental set up with some key differences: firstly, the substrate factor was removed by maintaining all treatments at the same (rich) conditions. Secondly, in order to allow for the removal of predators in the alternating treatment, we froze portions of all the experimental cultures at the end of each growth cycle in a 1:1 (vol:vol) mix with glycerol, and used these frozen stocks as inocula for the following cycle.

Isolates from the treatment with alternating predator presence showed still sensitivity to the presence of the flagellate after 20 growth cycles: higher aggregation of bacteria was observed if they were grown in the supernatants of a predator-prey co-culture. This finding experimentally supports the theoretically derived conclusion that unstable environmental conditions help to maintain phenotypic plasticity (Yamamichi et al. 2011). By contrast, strains evolved with constant presence or absence of the predator lost their plasticity. This also conformed with theoretical considerations, as reduced plasticity can have a fitness advantage in stable environmental conditions due to the innate costs of keeping phenotypical flexibility (Callahan et al. 2008).

Another effect of the co-cultivation either at continuous or alternating presence of a predator was that these strains could grow to higher total cell densities in co-culture with a flagellate than the ancestor or the strain evolved without predation. It was shown previously that grazing by protists can enhance bacterial growth rates (Posch et al. 1999). This might be due to reduction of intraspecific competition (Hahn and Höfle 2001) or that the predators release additional substrates, which promote bacterial growth (Caron et al. 1988). It is therefore conceivable that the high mortality selected for enhanced growth rates maybe at the cost of the growth efficiency. This possible disadvantage would explain, why such growth adapted strains were not favoured by selection in the treatment evolved without predator.

5.2 Manuscript 3

Adaptive phenotypic defence is only preserved in a bacterial strain at alternating conditions of predator presence and absence

Adaptive phenotypic defence is only preserved in a bacterial strain at alternating conditions of predator presence and absence

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Abstract

Many organisms feature subpopulations of so-called defence phenotypes that are fully or partially protected from predation. Such variation may either be a randomly fixed trait, or the defence phenotype may be specifically induced by external triggers. Theoretical analysis of eco-evolutionary predator-prey dynamics predicts that constant environments should select for the loss of phenotypic plasticity due to its inherent fitness costs and favour genotypically fixed trait variation. By contrast, adaptive formation of defence phenotypes should be preserved at fluctuating environmental conditions. We set up an evolutionary experiment to test these predictions. A chemical cue from a bacterivorous flagellate triggers the production of grazing-protected cell aggregates in the freshwater bacterium *Sphingobium* sp. Z007. This defence trait was either constitutively enhanced or reduced in isolates that had been grown for 20 cycles with or without predators, respectively. Moreover, the so evolved strains had lost the ability to increase aggregate formation in supernatants of predator-prey co-cultures. However, if the flagellate predators were only present at every second growth cycle, the proportions of the grazing-protected subpopulation of the evolved strains were indistinguishable from that of the ancestor. In addition, the ability to adapt to the presence of predators was conserved in these isolates. Our results thus provide direct experimental evidence for the evolutionary mechanism that was proposed to select for either fixed trait variability or phenotypic plasticity.

Introduction

Free-living bacteria often face highly variable environmental condition with drastic short-term changes of temperature, light, pH, or nutrient concentrations. While this is arguably most pronounced in terrestrial realms (Ramette and Tiedje 2007), spatial heterogeneity may also be important in seemingly unstructured aquatic habitats (Salcher et al. 2011, Stocker 2012). Seasonally dependent fluctuations, such as weather conditions or the successions of different plankton species (Posch et al. 2015), as well as transient spatial gradients (Wuest and Lorke 2003) influence the abundances and activities of aquatic microorganisms. On an even smaller scale, the immediate surrounding of bacteria can display a high degree of heterogeneity: Macroscopic organic aggregates are hot-spots for microbial growth and diversity (Kiørboe and Jackson 2001, Simon et al. 2002), and turbulent stirring may lead to their transient containment in zones of high competition and predation (Durham et al. 2013).

Thus, the ability to perceive and process external signals is an essential trait in dynamic environments, resulting in enhanced adaptability to fluctuating conditions (Wadhams and Armitage 2004). For example a chemotactic system allows many bacterial species to detect gradients of either positive or negative stimuli (Armitage 2001). Similar receptors are used to perceive other signals that influence their metabolic state; extrinsic factors such as oxygen, light or the magnetic field help to determine the availability of energy or the redox state, or to find the optimal position in a physical gradient (Taylor et al. 1999). The determination of the numbers of surrounding kin (quorum sensing) allows for density-dependent cooperative behaviour such as the formation of biofilms (Davies et al. 1998), swarming motility (Köhler et al. 2000) or expression of virulence factors (Novick 2003).

Signal perception can also play a crucial role between species in microbial predator-prey interactions. Due to the inherent fitness costs of permanently expressing defence traits (Steiner 2007), microorganisms may be able to conditionally switch between growth optimised but vulnerable and grazing protected forms. For example, the cyanobacterium *Microcystis aeruginosa* increased the expression of the toxic heptapeptide microcystin in the presence of a daphnid predator (Pineda-Mendoza et al. 2014). Upon sensing the presence of a bacterivorous nanoflagellate a *Flectobacillus* sp. changed its cell shapes into inedible filaments (Corno and Jürgens 2008).

However, environmental perception might only be maintained under conditions that give a selective advantage to this feature; an invariable environment is believed to lead to genotypic specialization, resulting in either the loss, permanent downregulation or constitutive expression of the conditionally expressed trait (Yamamichi et al. 2011). *Bacillus subtilis* cultivated under constant and sporulation-repressing conditions lost its innate ability to sporulate but also motility; this was, moreover, accompanied by a reduced transcription of genes that are important to perceive and react to signals from the environment (Maughan and Nicholson 2011). Social traits in *Myxococcus xanthus* were lost when evolving under constant conditions that render social behaviour superfluous (Velicer et al. 1998).

The freshwater isolate *Sphingobium* sp. Z007 has two distinct phenotypic states, free planktonic cells and grazing-protected aggregates (Blom et al. 2010a). These bacteria are able to sense the presence of a protistan predator; an unidentified infochemical serves as a signal to enhance cell aggregation (Blom et al. 2010b). The formation of such cell clusters is thought to be associated with additional metabolic costs such as the secretion of an exopolymeric matrix and higher intraspecific 'shadow' competition (Lubin et al. 2001) within the aggregate. In a previous study we propagated *Sphingobium* sp. Z007 at reduced substrate concentrations for 29 batch growth cycles either with or without a bacterivorous flagellate (Baumgartner et al. 2016a). Depending of the treatment, the so evolved strains exhibited a constitutively enhanced or reduced production of cell aggregates, and they had lost the ability to sense flagellates in supernatants of spent predator-prey co-cultures. We, therefore, hypothesised that the cultivation of *Sphingobium* sp. Z007 at alternating states of predator presence or absence should preserve the ancestral sensing trait, whereas constant conditions of either state should lead to its loss.

Materials and Methods

Bacterial and flagellate strains

The bacterial strain, *Sphingobium* sp. Z007 belongs to the family of *Sphingomonadaceae* (α subgroup of proteobacteria) and has originally been isolated from Lake Zurich. Pronounced formation of cell aggregates is induced in this strain in

the presence of a flagellate predator, and this behaviour is triggered by the sensing of an unknown infochemical (Blom et al. 2010b). The mixotrophic nanoflagellate *Poterioochromonas* sp. strain DS (Blom et al. 2010a) served as a predator. It was maintained axenically at 18°C in the dark, reinoculated biweekly into fresh *Ochromonas* medium (Culture collection of algae (SAG)) and additionally fed with heat killed cells of *Flectobacillus major* DSMZ 103. Prior to the experiment the bacterial strain was taken from cryo-stock cultures stored at -80°C, inoculated into DSMZ 7 medium (1 g L^{-1} yeast extract, 1 g L^{-1} glucose, and 1 g L^{-1} peptone; German Collection of Microorganisms and Cell Cultures [DSMZ], Braunschweig, Germany) and grown in batch culture for 72h at 18°C in the dark.

Alternating predator presence experiment

To test the effect of alternating predator presence we set up a sequential batch culture experiment with three different treatments: two triplicates sets of cultures were always grown with or without predators, respectively (treatments P+ and P-), and a third set had alternating batch growth cycles in the presence and absence of predators ('fluctuating' treatment F). Microbes were grown in 50 ml of DSMZ 7 medium in the dark at 18°C. Every batch incubation cycle lasted for 72h, and samples were taken every 24h for subsequent flow cytometric analysis of cell densities (see below). In order to quantitatively remove predators in treatment F between cycles, subsamples of 500 µl were taken after 72 h of growth, diluted with an equal volume of glycerol and stored overnight at -80°C. This procedure was applied to all replicates from all three treatments irrespective of the actual presence of flagellates. For the next growth cycle, bacteria were re-inoculated into fresh DSMZ 7 medium from overnight cryo-stocks at initial densities of approximately $3x10^5$ cells ml^{-1}. Additionally, 10^4 flagellate cells ml^{-1} were added to the P+ and the F treatment at the beginning of each cycle, or of each second cycle, respectively. The experiment ran for 20 growth cycles (60 days), which was equivalent to approximately 300 bacterial generations in the P- treatment. After this period, subsamples from each replicate of each treatment were streaked on agar plates containing DSMZ 7 medium. Three colonies were isolated from each plate and stored in a 1:1 mix of DSMZ 7 medium and glycerol at -80°C for the subsequent growth experiments. Prior to all experiments described below the strains from frozen stock cultures were first inoculated into DSMZ 7 medium and incubated for 72h at 18°C.

Growth and biofilm formation of isolates

The ancestral strain and the evolved isolates were inoculated in triplicates in a 96 well plate containing 200 µl of DSMZ 7 medium at initial densities of $1x10^5$ cells per well. The plate was incubated in an absorption plate reader (molecular devices, SpectraMax 190) for 72h at 18°C, and optical density at 600 nm (OD_{600}) was measured every 30 min.

After 72 h of growth, 20 µl of a 0.1% crystal violet staining solution was added to each well to determine the intensity of biofilm formation. After 10 min of incubation the liquid phase was removed and the wells were washed twice with PBS and air dried. Next, 200 µl of 100% ethanol was added to each well to re-dissolve the stain. After 15 min of incubation OD_{600} was measured on an absorption plate reader (molecular devices, SpectraMax 190).

Predator prey co-culture and supernatant experiments

The ancestor strain and the evolved isolates were incubated in triplicates either with or without predator in 50 ml of DSMZ 7 medium at 18°C in the dark. Initial bacterial and flagellate densities were $3x10^5$ ml^{-1} and $1x10^4$ ml^{-1}, respectively. Samples were taken every 24h for 72 h and cell densities were determined by flow cytometer. At the end of the experiments, cells were removed from the cultures of the ancestral strain (grown with and without predators) by centrifugation (2x at 8000 rpm). The culture supernatants were transferred to fresh tubes and stored at -21°C until further processing.

For experiments on aggregate induction by a chemical cue, the supernatants obtained from the above described bacterial cultures and bacteria-flagellate co-cultures were thawed and immediately transferred into a 24-well plate (2 ml per well). Thereafter, the ancestral and evolved strains were inoculated in triplicates into the supernatants at an initial cell density of $3x10^5$ cells ml^{-1}. Bacteria were incubated in the supernatants for 48 h at 18°C in the dark and samples for flow cytometry were taken at the end of the experiment.

Flow cytometry

All samples for flow cytometry were fixed with glutaraldehyde (2.5% final concentration), and stained with 4',6-diamidino-2-phenylindole (DAPI, final concentration, 1 µg ml^{-1}). Abundances of bacteria and flagellates were measured on an Influx V-Gs cell sorter (Becton Dickinson Inc., San Jose, CA) equipped with a UV (350 nm) and blue (488 nm) laser. Flow cytometric data were then evaluated with the custom software JoFlow (Villiger and Pernthaler, unpublished). Free cells of *Sphingobium* sp. Z007 were identified in dot plots of side scattered blue light (SSC) versus 460 nm (DAPI fluorescence). Due to its larger cell size and different cellular composition the predator *Poterioochromonas* sp. strain DS was detected by using the scattered light at a wavelength of 531 nm versus 460 nm. Aggregates were defined as events in the cytogram, which have equal or higher fluorescence properties as the flagellate.

Results

Experiment with alternating predator presence

Over the first six growth cycles the ratio of aggregates to free cells (a/f) was slightly higher in P+ than in the other treatments (Fig. 1). Thereafter, the differences between the treatments became substantially more pronounced: A clear adaptation to continuous predation pressure of bacteria in P+ was reflected by increasingly higher a/f, reaching a maximum after cycle 12. By contrast, the strains cultivated without predator (P-) showed consistently low a/f. In treatment F the ratio of aggregates to free cells was strongly related to the presence of *Poterioochromonas*: a/f was high in cycles with bacteria-flagellate co-cultures and low otherwise.

Growth curve and biofilm staining

Batch culture growth characteristics (growth rate during exponential phase, length of the lag phase) and biofilm formation strongly differed between isolates from the P+ and P- treatments (Fig. 2). Strains evolved without predation reached the highest stationary phase OD (Fig. 2A), but formed the lowest amount of biofilm (Fig. 2B), whereas the opposite was observed for strains originating from P+. The ancestor

Sphingobium sp. Z007 and the strains isolated from the F treatment had nearly identical growth curves and also formed similar amounts of biofilm.

C*o-cultures of ancestor and evolved strains with predators*

To compare growth and grazing resistance we inoculated the ancestor strain and evolved isolates with and without flagellate predators. In the treatments without predators the numbers of free cells was similar in the ancestor and isolates from the P- and F treatments (Fig. 3A), while P+ strains showed a significant lower cell density than all other strains. The lower abundances of free cells in the latter strains was counterbalanced by an elevated density of aggregates, which was more than 12 times higher than in the other strains (Fig. 3B). In the presence of *Poterioochromonas* the ancestor strain and the isolates evolved without predator clearly suffered from grazing, resulting in lower densities of free cells (Fig. 3 C). By contrast, the numbers of free cells were least affected by flagellate grazing in strains evolved under fluctuating conditions, which grew to almost an order of magnitude higher densities than the P- strains. Aggregate density was generally higher in the treatment with predators (Fig. 3D). Differences between the two treatments were most pronounced in the ancestor strain and in isolates evolved under fluctuating conditions, which both formed 4 to 5 times more aggregates if grown together with flagellates than in pure culture.

Induction of aggregate formation by supernatants

To test if a chemical factor from a predator-prey co-culture had an aggregate inducing effect on the evolved isolates, the ancestor strain and strains with different evolutionary background were inoculated into supernatants of spent cultures of *Sphingobium* sp. Z007 grown with or without flagellates. Strains evolved under constant conditions (P+, P-) showed no difference in the numbers of aggregates between the two types of supernatants (Fig. 4). By contrast, both, the ancestor strain and the isolates from the F treatment had increased aggregate formation in supernatant of the predator-prey co-culture.

Discussion

Many environments demand special adaptations to cope with ever-changing and in parts unpredictable conditions. Some bacteria show phenotypic variability even in clonal populations (Elowitz et al. 2002). This trait helps to quickly adapt to dynamic conditions, and is thus widely recognized for its ecological benefits (Scheiner 1993). A permanent state of population heterogeneity can be, e.g., realized by stochastic expression patterns of particular genes (Raj and van Oudenaarden 2008). Alternatively, phenotypic variability may be adaptive in response to a variety of external triggers, e.g. altered growth conditions (Hahn et al. 1999) or specific infochemicals (Corno and Jürgens 2006, Blom et al. 2010b); such plasticity has, moreover, a stabilizing effect on community dynamics (Kovach-Orr and Fussmann 2013). The resulting bistability into phenotypically distinct subpopulations is suggested to be a risk spreading, 'bet-hedging' strategy in order to maximize survival (Edwards 2012). Phenotypic variability may also be favourable for microbes in aquatic environments where fundamental survival factors can change on a time scale of few generations. For example, algal blooms first provide substrates for rapid bacterial proliferation, but are accompanied by the raise of protistan predators (Eckert et al. 2012). This may rapidly shift the control of bacterioplankton growth from bottom-up to top-down limitation (Psenner and Sommaruga 1992), leading to the accumulation of grazing-protected bacterial phenotypes such as filaments or cell aggregates (Jürgens and Güde 1994).

The studied *Sphingobium* sp. strain Z007 has been isolated from the central part of a large lake by addition of both, substrates and predators (Blom and Pernthaler 2010). Its closest relatives originate from terrestrial habitats, which suggests that these bacteria may regularly traverse between biomes with very contrasting selective properties. The strain has a motile planktonic form and readily forms colonies of biofilms on solid surfaces. It always features a subpopulation of aggregated cells, the size of which depends on the growth state of the culture (Blom et al. 2010a). In addition, it is able to respond by increased aggregation to a conspecific chemical cue that is released in the presence of *Poterioochromonas* (either by bacteria or flagellates) (Blom et al. 2010b). Aggregate formation in these bacteria thus appears to be triggered by a mix of extrinsic signals, random switching and physiological state, which has been

interpreted as a strategy to exist in the face of unreliable environmental signals (Arnoldini et al. 2012).

The aggregated phenotype was clearly selected for or against if bacteria were evolved in the permanent presence or absence of predators (Fig 1, 3A). Moreover, there was a relationship between the extent of aggregation and biofilm formation: the latter trait was also significantly enhanced or reduced in strains from the P+ and P- treatments, respectively (Fig 2B). So-called protobiofilms -bacteria attached to suspended microscopic flocs of exopolymers– may function as the seeds for biofilms in coastal marine waters (Bar-Zeev et al. 2012). Therefore, it is likely that aggregation and biofilm formation are closely related phenotypes, e.g., both involve the loss of motility, the production of exopolymers, or the expression of type 4 secretion systems (Ghigo 2001, Flemming and Wingender 2010) (all features that are present in the genome of the studied strain (Baumgartner et al. 2016b)).

The abundance of aggregates in isolates from P+ was always high even if no flagellates were present, whereas isolates from the P- treatment formed only few aggregates even in predator-prey co-cultures (Fig 3). The evolved *Sphingobium* strains, moreover, had lost their ability to sense the predators in order to phenotypically adapt (Fig.4). This is evidence for the genotypic fixation of the respective proportions of the growth or defence phenotypes, i.e., a rapid, almost contemporary evolutionary process in the context of an ecological interaction (or the lack thereof) (Thompson 1998, Carroll et al. 2007). The shifts in the proportions of planktonic and aggregated subpopulations in isolates from P+ and P- replicate findings from our earlier experiment where both, predation and substrate supply were manipulated simultaneously (Baumgartner et al. 2016a). In contrast to that study, our current experimental setup precluded any evolutionary feed-back between predators and prey: *Poterioochromonas* was first eliminated by freezing during each growth cycle and then re-inoculated from a culture that was fed with another bacterial strain. Thus, the observed phenotypic adaptations of *Sphingobium* were unaffected by both, substrate levels or potential co-evolutionary dynamics. A single factor, the permanent presence or absence of flagellates, was sufficient to elicit this response.

Our findings provide experimental evidence for a prediction by Yamamichi et al (Yamamichi et al. 2011): Based on a theoretical analysis the authors concluded that the specialist genotypes in polymorphic populations should always outcompete the

phenotypically plastic one at constant selective conditions if environmental perception is associated with a fitness cost, e.g., with respect to production or maintenance (DeWitt et al. 1998). Being a sequential batch culture, our experimental system did not strictly adhere to the premise of constancy, ideally realized only by chemostat cultivation (Arndt et al. 1992, Corno and Jürgens 2008). Nevertheless, it clearly reproduced the predicted evolutionary shift, likely because the inocula for the subsequent growth cycle always originated from a situation in which bacterial population sizes were either limited by substrates or predators (Fig.1). The alternating presence and absence of the predator (Fig. 1+2) resulted in the conservation of phenotypic plasticity in *Sphingobium* isolates evolved under such conditions (Fig. 4). Moreover, the growth of these strains, their respective proportions of free cells and aggregates in the absence of predators and their ability to form biofilms were indistinguishable from the ancestral strain (Figs. 2, 3). Again, this is experimental evidence for the model of Yamamichi et al (Yamamichi et al. 2011) that allows for the conservation of adaptive phenotypic plasticity under fluctuating environmental conditions.

A significantly higher number of free cells of isolates from the F and P+ treatments survived in the presence of the predators (Fig. 3). In general, the grazing by heterotrophic nanoflagellates can enhance bacterial growth rates (Posch et al. 1999, Šimek et al. 2001) due to a decrease of intraspecific competition (Hahn, 2001), or the release of additional substrates or nutrients by the predator (Caron, 1988). However, in our case, this response appeared to be a specific consequence of the experimental treatment. There are two possible interpretations for the enhanced growth of free cells in isolates evolved with predators. For one, it is conceivable that the high mortality rates selected for genotypes that reproduced more rapidly in their planktonic state than the ancestor, possibly at the cost of growth efficiency. The latter would explain why such genotypes were not selected for in the absence of predators: As outlined above, the starter populations for each growth cycle of the experiment were obtained from stationary phase cultures, which in P- likely included a large proportion of cells that had reproduced at increasingly substrate-limiting condition. Alternatively, it is conceivable that free cells were continuously released from aggregates, as is known for biofilms (Stoodley et al. 2001). However, the latter explanation does not account for the observation that there were similar numbers of free cells in P+ and F

isolates in the presence of flagellates even though their respective numbers of aggregates differed by a factor of 4. (Fig. 3).

In summary, our experiments show that adaptive phenotypic plasticity could only be conserved in the studied bacteria by alternating periods of growth with and without flagellate predators. By contrast, the permanent presence or absence of predation rapidly led to an evolutionary bifurcation into strains that predominantly invested in the free-living (growth) or aggregated (defence) phenotype, respectively.

References:

Armitage, J. P. 2001. Bacterial Taxis. eLS. John Wiley & Sons, Ltd.

Arndt, H., H. Güde, M. Macek, and K. O. Rothhaupt. 1992. Chemostats used to model the microbial food web: evidence for the feedback effect of herbivorous metazoans. Pages 187-194 *in* Microbial ecology of pelagic environments.

Arnoldini, M., R. Mostowy, S. Bonhoeffer, and M. Ackermann. 2012. Evolution of Stress Response in the Face of Unreliable Environmental Signals. PLoS Comput Biol **8**:e1002627.

Bar-Zeev, E., I. Berman-Frank, O. Girshevitz, and T. Berman. 2012. Revised paradigm of aquatic biofilm formation facilitated by microgel transparent exopolymer particles. Proceedings of the National Academy of Sciences **109**:9119-9124.

Baumgartner, M., T. R. Neu, J. F. Blom, J. Pernthaler. 2016 Predation on a bacterial strain outweighs substrate limitation in selecting for defence over growth optimized morphotypes. (Submitted)

Baumgartner, M., S. Roffler, T. Wicker, J.F. Blom, J. Pernthaler. 2016. Letting go: genome streamlining allows for simultaneous adaptation to top-down and bottom-up constraints in a phenotypically plastic bacterial strain. (Submitted)

Blom, J. F., K. Horňák, K. Šimek, and J. Pernthaler. 2010a. Aggregate formation in a freshwater bacterial strain induced by growth state and conspecific chemical cues. Environmental Microbiology **12**:2486-2495.

Blom, J. F. and J. Pernthaler. 2010. Antibiotic effects of three strains of chrysophytes (Ochromonas, Poterioochromonas) on freshwater bacterial isolates. FEMS Microbiology Ecology **71**:281-290.

Blom, J. F., Y. S. Zimmermann, T. Ammann, and J. Pernthaler. 2010b. Scent of Danger: Floc Formation by a Freshwater Bacterium Is Induced by Supernatants from a Predator-Prey Coculture. Applied and Environmental Microbiology **76**:6156-6163.

Caron, D. A., J. C. Goldman, and M. R. Dennett. 1988. Experimental demonstration of the roles of bacteria and bacterivorous protozoa in plankton nutrient cycles. Hydrobiologia **159**:27-40.

Carroll, S. P., A. P. Hendry, D. N. Reznick, and C. W. Fox. 2007. Evolution on ecological time-scales. Functional Ecology **21**:387-393.

Corno, G. and K. Jürgens. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. Applied and Environmental Microbiology **72**:78-86.

Corno, G. and K. Jürgens. 2008. Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. Environmental Microbiology **10**:2857-2871.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm. Science **280**:295-298.

DeWitt, T. J., A. Sih, and D. S. Wilson. 1998. Costs and limits of phenotypic plasticity. Trends in Ecology & Evolution **13**:77-81.

Durham, W. M., E. Climent, M. Barry, F. De Lillo, G. Boffetta, M. Cencini, and R. Stocker. 2013. Turbulence drives microscale patches of motile phytoplankton. Nature communications **4**.

Eckert, E. M., M. M. Salcher, T. Posch, B. Eugster, and J. Pernthaler. 2012. Rapid successions affect microbial N-acetyl-glucosamine uptake patterns during a lacustrine spring phytoplankton bloom. Environmental Microbiology **14**:794-806.

Edwards, A. M. 2012. Phenotype Switching Is a Natural Consequence of Staphylococcus aureus Replication. Journal of Bacteriology **194**:5404-5412.

Elowitz, M. B., A. J. Levine, E. D. Siggia, and P. S. Swain. 2002. Stochastic Gene Expression in a Single Cell. Science **297**:1183-1186.

Flemming, H.-C. and J. Wingender. 2010. The biofilm matrix. Nature Reviews Microbiology **8**:623-633.

Hahn, M. W. and M. G. Höfle. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiology Ecology **35**:113-121.

Ghigo, J.-M. 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature **412**:442-445.

Hahn, M. W., E. R. Moore, and M. G. Höfle. 1999. Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. Applied and Environmental Microbiology **65**:25-35.

Jürgens, K. and H. Güde. 1994. The potential importance of grazing-resistant bacteria in planktonic systems. Marine Ecology Progess Series **112**:169-188.

Kiørboe, T. and G. A. Jackson. 2001. Marine snow, organic solute plumes, and optimal chemosensory behavior of bacteria. Limnology and Oceanography **46**:1309-1318.

Köhler, T., L. K. Curty, F. Barja, C. van Delden, and J.-C. Pechère. 2000. Swarming of Pseudomonas aeruginosa Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. Journal of Bacteriology **182**:5990-5996.

Kovach-Orr, C. and G. F. Fussmann. 2013. Evolutionary and plastic rescue in multitrophic model communities. Philosophical Transactions of the Royal Society of London B: Biological Sciences **368**.

Lubin, Y., J. R. Henschel, and M. B. Baker. 2001. Costs of aggregation: shadow competition in a sit-and-wait predator. Oikos **95**:59-68.

Maughan, H. and W. L. Nicholson. 2011. Increased Fitness and Alteration of Metabolic Pathways during Bacillus subtilis Evolution in the Laboratory. Applied and Environmental Microbiology **77**:4105-4118.

Novick, R. P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Molecular Microbiology **48**:1429-1449.

Pineda-Mendoza, R. M., G. Zúñiga, and F. Martínez-Jerónimo. 2014. Infochemicals released by Daphnia magna fed on Microcystis aeruginosa affect mcyA gene expression. Toxicon **80**:78-86.

Posch, T., B. Eugster, F. Pomati, J. Pernthaler, G. Pitsch, and E. Eckert. 2015. Network of Interactions Between Ciliates and Phytoplankton During Spring. Frontiers in Microbiology **6**.

Posch, T., K. Simek, J. Vrba, J. Pernthaler, J. Nedoma, B. Sattler, B. Sonntag, and R. Psenner. 1999. Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. Aquatic Microbial Ecology **18**:235-246.

Psenner, R. and R. Sommaruga. 1992. Are rapid changes in bacterial biomass caused by shifts from top-down to bottom-up control? Limnology and Oceanography **37**:1092-1100.

Raj, A. and A. van Oudenaarden. 2008. Stochastic gene expression and its consequences. Cell **135**:216-226.

Ramette, A. and J. M. Tiedje. 2007. Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. Proceedings of the National Academy of Sciences of the United States of America **104**:2761-2766.

Salcher, M. M., J. Pernthaler, N. Frater, and T. Posch. 2011. Vertical and longitudinal distribution patterns of different bacterioplankton populations in a canyon-shaped, deep prealpine lake. Limnology and Oceanography **56**:2027-2039.

Scheiner, S. M. 1993. Genetics and Evolution of Phenotypic Plasticity. Annual Review of Ecology and Systematics **24**:35-68.

Šimek, K., J. Pernthaler, M. G. Weinbauer, K. Hornák, J. R. Dolan, J. Nedoma, M. Mašín, and R. Amann. 2001. Changes in Bacterial Community Composition and Dynamics and Viral Mortality Rates Associated with Enhanced Flagellate Grazing in a Mesoeutrophic Reservoir. Applied and Environmental Microbiology **67**:2723-2733.

Simon, M., H. P. Grossart, B. Schweitzer, and H. Ploug. 2002. Microbial ecology of organic aggregates in aquatic ecosystems. Aquatic Microbial Ecology **28**:175-211.

Steiner, U. K. 2007. Investment in defense and cost of predator-induced defense along a resource gradient. Oecologia **152**:201-210.

Stocker, R. 2012. Marine Microbes See a Sea of Gradients. Science **338**:628-633.

Stoodley, P., S. Wilson, L. Hall-Stoodley, J. D. Boyle, H. M. Lappin-Scott, and J. W. Costerton. 2001. Growth and Detachment of Cell Clusters from Mature Mixed-Species Biofilms. Applied and Environmental Microbiology **67**:5608-5613.

Taylor, B. L., I. B. Zhulin, and M. S. Johnson. 1999. Aerotaxis and Other Energy-Sensing Behavior in Bacteria. Annual Review of Microbiology **53**:103-128.

Thompson, J. N. 1998. Rapid evolution as an ecological process. Trends in Ecology & Evolution **13**:329-332.

Velicer, G. J., L. Kroos, and R. E. Lenski. 1998. Loss of social behaviors by Myxococcus xanthus during evolution in an unstructured habitat. Proceedings of the National Academy of Sciences **95**:12376-12380.

Wadhams, G. H. and J. P. Armitage. 2004. Making sense of it all: bacterial chemotaxis. Nat Rev Mol Cell Biol **5**:1024-1037.

Wuest, A. and A. Lorke. 2003. Small-scale hydrodynamics in lakes. Annual Review of Fluid Mechanics **35**:373-412.

Yamamichi, M., T. Yoshida, and A. Sasaki. 2011. Comparing the effects of rapid evolution and phenotypic plasticity on predator-prey dynamics. The American Naturalist **178**:287-304.

Figures

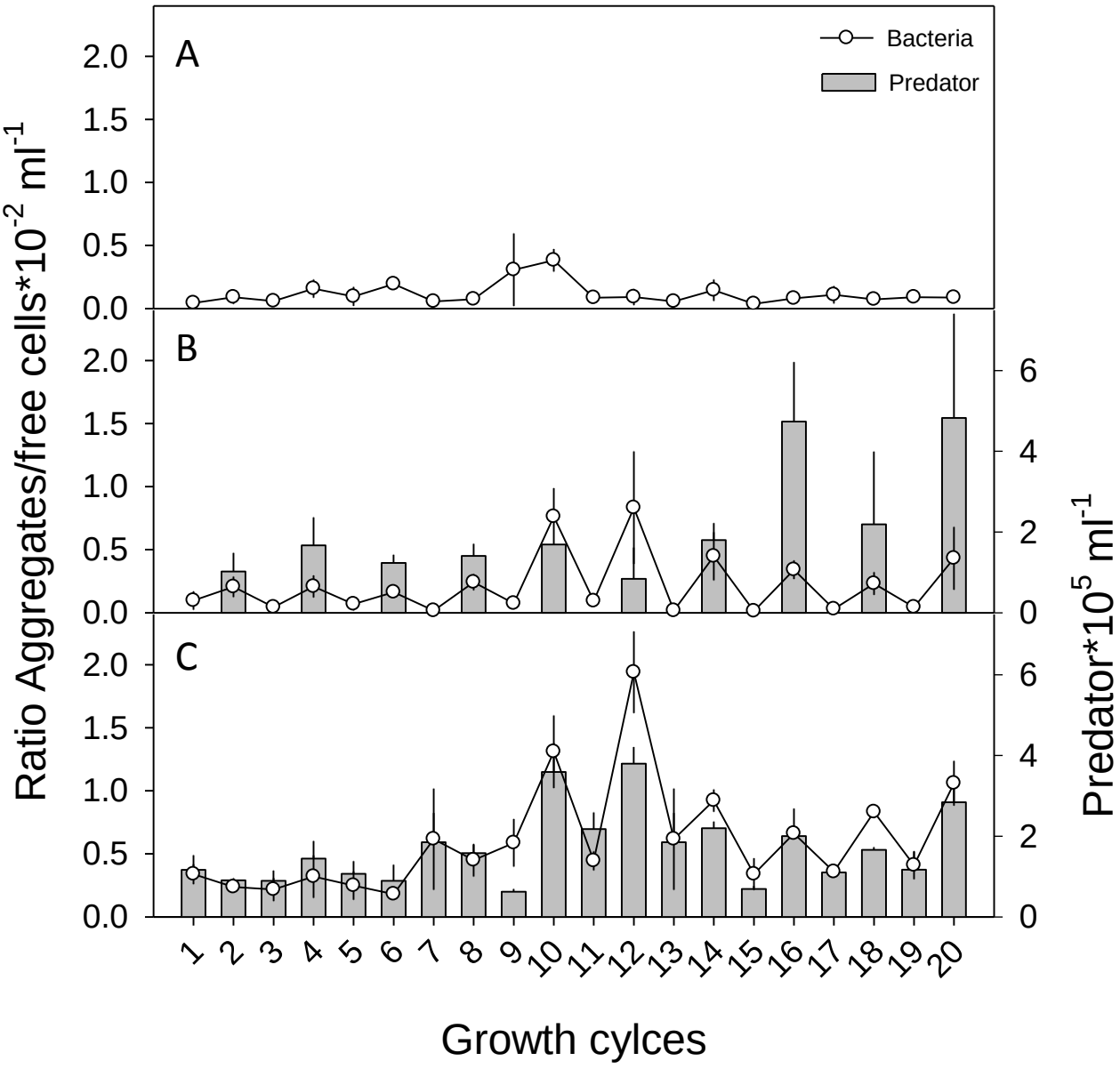

Fig. 1: Ratios of aggregated to free cells of *Sphingobium* sp. Z007 during 20 (3-day) growth cycles. Populations evolving (A) constantly without predators, (B) at alternating cycles of predator presence and absence and (C) constantly with predators. Error bars denote 1 standard deviation of 3 biological replicates.

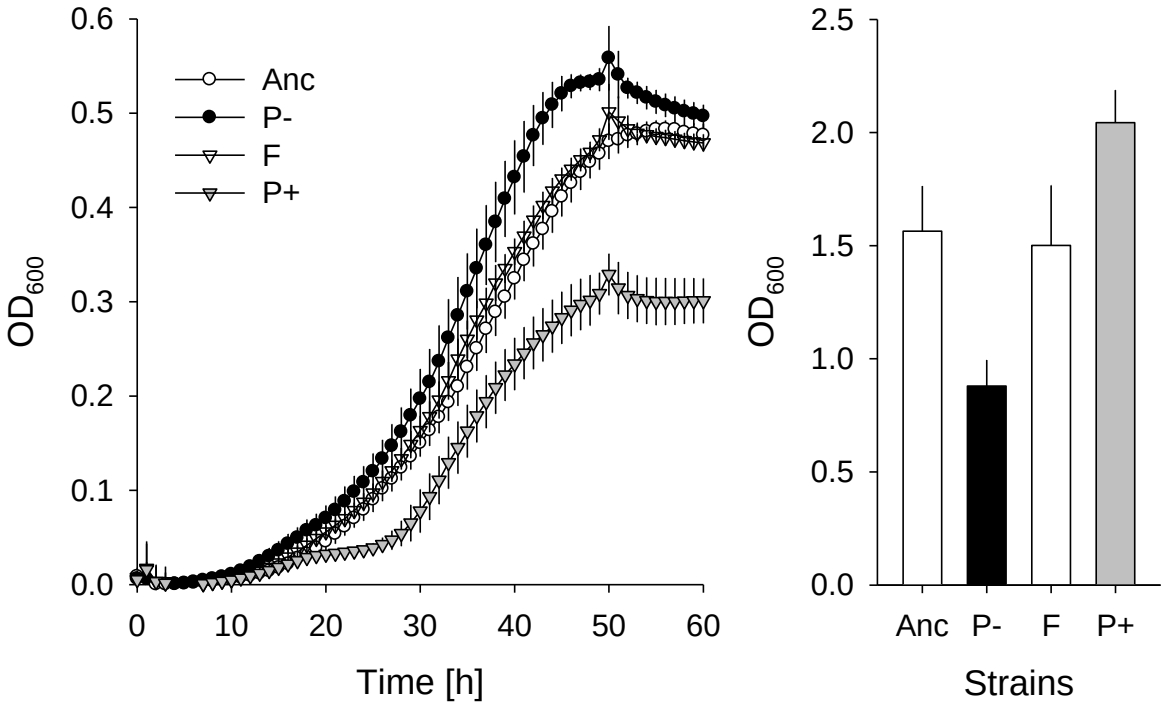

Fig. 2: (A) Growth curves of the ancestor strain (Anc), and of isolates evolved continuously with or without predators (P+, P-), or at alternating presence and absence of predator (F). (B) Intensity of biofilm formation of ancestor and evolved strains as determined by crystal violet staining. Error bars as in Fig. 1.

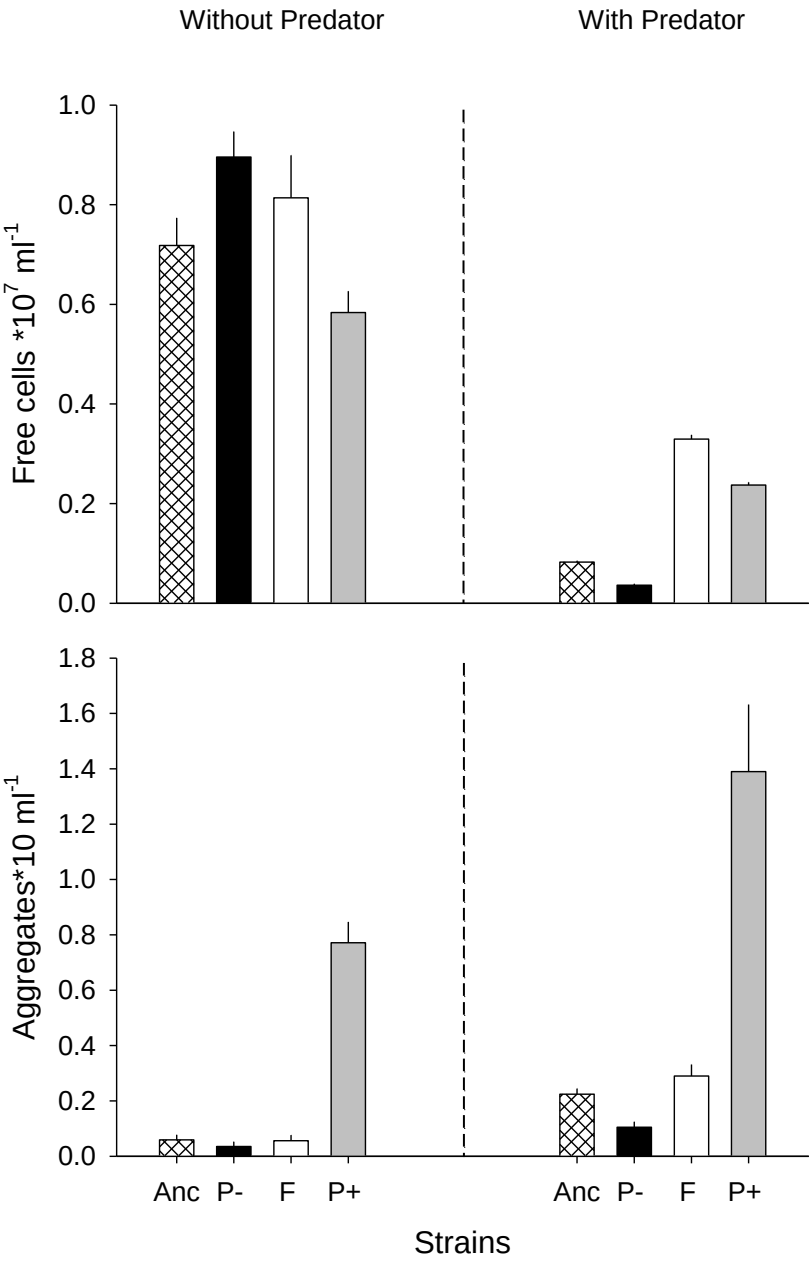

Fig. 3: Densities of planktonic cells and cell aggregates of the ancestor and the various evolved strains after 24 h of growth. A, B: cultivation in the absence of predators; C, D predator-prey cocultures. Error bars as in Fig. 1.

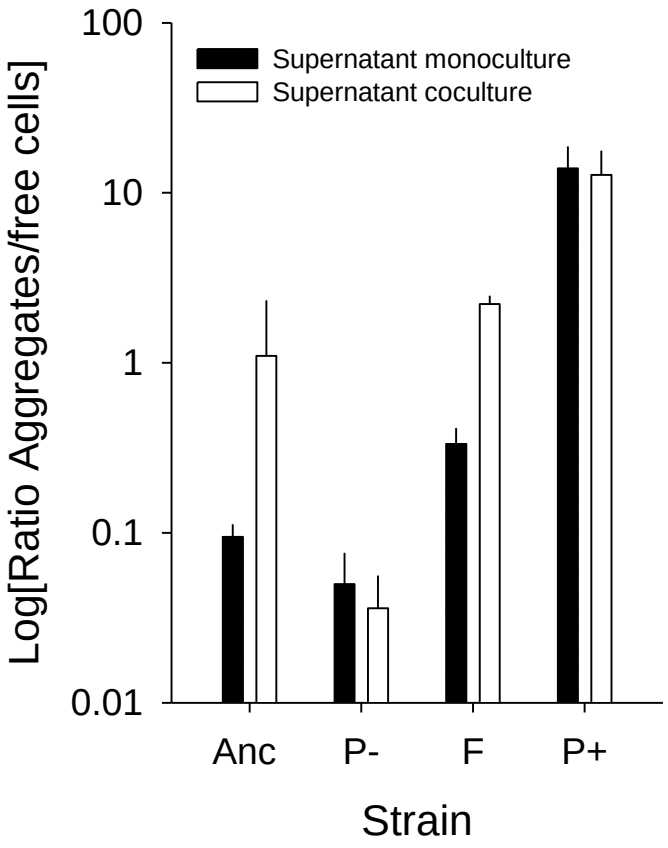

Fig. 4: Ratios of aggregated to free cells in the ancestor strain and the evolved isolates after 24 h exposure to supernatants of bacterial monocultures or bacteria-flagellate cocultures. Error bars as in Fig. 1.

6 References

Adams, J. and F. Rosenzweig. 2014. Experimental microbial evolution: history and conceptual underpinnings. Genomics **104**:393-398.

Alegado, R. A., L. W. Brown, S. Cao, R. K. Dermenjian, R. Zuzow, S. R. Fairclough, J. Clardy, and N. King. 2012. A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. eLife **1**:e00013.

Aylward, F. O., B. R. McDonald, S. M. Adams, A. Valenzuela, R. A. Schmidt, L. A. Goodwin, T. Woyke, C. R. Currie, G. Suen, and M. Poulsen. 2013. Comparison of 26 Sphingomonad Genomes Reveals Diverse Environmental Adaptations and Biodegradative Capabilities. Applied and Environmental Microbiology **79**:3724-3733.

Azam, F., T. Fenchel, J. G. Field, J. Gray, L. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. Estuaries **50**.

Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K.-i. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. The Lancet **359**:1819-1827.

Baharoglu, Z. and D. Mazel. 2014. SOS, the formidable strategy of bacteria against aggressions. Fems Microbiology Reviews **38**:1126-1145.

Barrick, J. E. and R. E. Lenski. 2013. Genome dynamics during experimental evolution. Nat Rev Genet **14**:827-839.

Basta, T., S. Buerger, and A. Stolz. 2005. Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. Microbiology **151**:2025-2037.

Batut, B., C. Knibbe, G. Marais, and V. Daubin. 2014. Reductive genome evolution at both ends of the bacterial population size spectrum. Nat Rev Micro **12**:841-850.

Beardsley, C., J. Pernthaler, W. Wosniok, and R. Amann. 2003. Are Readily Culturable Bacteria in Coastal North Sea Waters Suppressed by Selective Grazing Mortality? Applied and Environmental Microbiology **69**:2624-2630.

Beaumont, H. J. E., J. Gallie, C. Kost, G. C. Ferguson, and P. B. Rainey. 2009. Experimental evolution of bet hedging. Nature **462**:90-93.

Beemelmanns, C., A. Woznica, R. A. Alegado, A. M. Cantley, N. King, and J. Clardy. 2014. Synthesis of the Rosette-Inducing Factor RIF-1 and Analogs. Journal of the American Chemical Society **136**:10210-10213.

Bennett, A. F. and R. E. Lenski. 1993. Evolutionary adaptation to temperature II. Thermal niches of experimental lines of Escherichia coli. Evolution:1-12.

Berleman, J. E. and J. R. Kirby. 2009. Deciphering the hunting strategy of a bacterial wolfpack. Fems Microbiology Reviews **33**:942-957.

Bieber, D., S. W. Ramer, C.-Y. Wu, W. J. Murray, T. Tobe, R. Fernandez, and G. K. Schoolnik. 1998. Type IV Pili, Transient Bacterial Aggregates, and Virulence of Enteropathogenic Escherichia coli. Science **280**:2114-2118.

Biers, E. J., S. Sun, and E. C. Howard. 2009. Prokaryotic genomes and diversity in surface ocean waters: interrogating the global ocean sampling metagenome. Applied and Environmental Microbiology **75**:2221-2229.

Bjedov, I., O. Tenaillon, B. Gérard, V. Souza, E. Denamur, M. Radman, F. Taddei, and I. Matic. 2003. Stress-Induced Mutagenesis in Bacteria. Science **300**:1404-1409.

Blom, J. F., K. Horňák, K. Šimek, and J. Pernthaler. 2010a. Aggregate formation in a freshwater bacterial strain induced by growth state and conspecific chemical cues. Environmental Microbiology **12**:2486-2495.

Blom, J. F., Y. S. Zimmermann, T. Ammann, and J. Pernthaler. 2010b. Scent of Danger: Floc Formation by a Freshwater Bacterium Is Induced by Supernatants from a Predator-Prey Coculture. Applied and Environmental Microbiology **76**:6156-6163.

Boenigk, J. and H. Arndt. 2002. Bacterivory by heterotrophic flagellates: community structure and feeding strategies. Antonie van Leeuwenhoek **81**:465-480.

Boenigk, J., K. Pfandl, and P. J. Hansen. 2006. Exploring strategies for nanoflagellates living in a'wet desert'. Aquatic Microbial Ecology **44**:71-83.

Bohannan, B. and R. Lenski. 2000. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. Ecology Letters **3**:362-377.

Bridges, B. A. 2001. Hypermutation in bacteria and other cellular systems. Philosophical Transactions of the Royal Society B: Biological Sciences **356**:29-39.

Bryant, J., C. Chewapreecha, and S. D. Bentley. 2012. Developing insights into the mechanisms of evolution of bacterial pathogens from whole-genome sequences. Future microbiology **7**:1283-1296.

Bryson, V. and W. Szybalski. 1952. Microbial selection. Science **116**:45-51.

Buckling, A., Y. Wei, R. C. Massey, M. A. Brockhurst, and M. E. Hochberg. 2006. Antagonistic Coevolution with Parasites Increases the Cost of Host Deleterious Mutations. Proceedings: Biological Sciences **273**:45-49.

Callahan, H. S., H. Maughan, and U. K. Steiner. 2008. Phenotypic plasticity, costs of phenotypes, and costs of plasticity. Annals of the New York Academy of Sciences **1133**:44-66.

Canchaya, C., G. Fournous, S. Chibani-Chennoufi, M.-L. Dillmann, and H. Brüssow. 2003. Phage as agents of lateral gene transfer. Current Opinion in Microbiology **6**:417-424.

Caron, D. A., J. C. Goldman, and M. R. Dennett. 1988. Experimental demonstration of the roles of bacteria and bacterivorous protozoa in plankton nutrient cycles. Hydrobiologia **159**:27-40.

Casadesús, J. and D. Low. 2006. Epigenetic gene regulation in the bacterial world. Microbiology and Molecular Biology Reviews **70**:830-856.

Cavicchioli, R., M. Ostrowski, F. Fegatella, A. Goodchild, and N. Guixa-Boixereu. 2003. Life under nutrient limitation in oligotrophic marine environments: an eco/physiological perspective of Sphingopyxis alaskensis (formerly Sphingomonas alaskensis). Microbial Ecology **45**:203-217.

Chapelle, E., R. Mendes, P. A. H. M. Bakker, and J. M. Raaijmakers. 2016. Fungal invasion of the rhizosphere microbiome. ISME J **10**:265-268.

Chou, H.-H., H.-C. Chiu, N. F. Delaney, D. Segrè, and C. J. Marx. 2011. Diminishing returns epistasis among beneficial mutations decelerates adaptation. Science **332**:1190-1192.

Collins, S., D. Sueltemeyer, and G. Bell. 2006. Changes in C uptake in populations of Chlamydomonas reinhardtii selected at high CO2. Plant, cell & environment **29**:1812-1819.

Cooper, V. S., D. Schneider, M. Blot, and R. E. Lenski. 2001. Mechanisms Causing Rapid and Parallel Losses of Ribose Catabolism in Evolving Populations of Escherichia coli B. Journal of Bacteriology **183**:2834-2841.

Cooper, V. S., S. H. Vohr, S. C. Wrocklage, and P. J. Hatcher. 2010. Why Genes Evolve Faster on Secondary Chromosomes in Bacteria. PLoS Comput Biol **6**:e1000732.

Cordero, O. X. and M. F. Polz. 2014. Explaining microbial genomic diversity in light of evolutionary ecology. Nature Reviews Microbiology **12**:263-273.

Corno, G. and K. Jürgens. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. Applied and Environmental Microbiology **72**:78-86.

Costa, R., I. M. Van Aarle, R. Mendes, and J. D. Van Elsas. 2009. Genomics of pyrrolnitrin biosynthetic loci: evidence for conservation and whole-operon mobility within Gram-negative bacteria. Environmental Microbiology **11**:159-175.

Coyne, J. A. and H. A. Orr. 2004. Speciation. Sinauer Associates Sunderland, MA.

Dallinger, W. 1878. On the life-history of a minute septic organism: with an account of experiments made to determine its thermal death point. Proceedings of the Royal Society of London **27**:332-350.

De Varigny, H. 1892. Experimental evolution. Lectures delivered in the Summer school of Art and Science. Edinburgh: University Hall (August 1891), London-New York: Mac Millan.

Dhar, R., R. Sägesser, C. Weikert, J. Yuan, and A. Wagner. 2011. Adaptation of Saccharomyces cerevisiae to saline stress through laboratory evolution. Journal of evolutionary biology **24**:1135-1153.

Dini-Andreote, F., F. Andreote, W. Araújo, J. Trevors, and J. van Elsas. 2012. Bacterial Genomes: Habitat Specificity and Uncharted Organisms. Microbial Ecology **64**:1-7.

Fox, J. W. and R. E. Lenski. 2015. From here to eternity—the theory and practice of a really long experiment. PLoS Biol **13**:e1002185.

Gasol, J. M., C. Pedrós-Alió, and D. Vaqué. 2002. Regulation of bacterial assemblages in oligotrophic plankton systems: results from experimental and empirical approaches. Antonie van Leeuwenhoek **81**:435-452.

Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman, and Å. Hagström. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Applied and Environmental Microbiology **65**:4475-4483.

Ghigo, J.-M. 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature **412**:442-445.

Gil, R., F. J. Silva, J. Peretó, and A. Moya. 2004. Determination of the Core of a Minimal Bacterial Gene Set. Microbiology and Molecular Biology Reviews **68**:518-537.

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field. 1990. GENETIC DIVERSITY IN SARGASSO SEA BACTERIOPLANKTON. Nature **345**:60-63.

Giovannoni, S. J., J. Cameron Thrash, and B. Temperton. 2014. Implications of streamlining theory for microbial ecology. ISME J **8**:1553-1565.

Giovannoni, S. J., H. J. Tripp, S. Givan, M. Podar, K. L. Vergin, D. Baptista, L. Bibbs, J. Eads, T. H. Richardson, M. Noordewier, M. S. Rappe, J. M. Short, J. C. Carrington, and E. J. Mathur. 2005. Genome streamlining in a cosmopolitan oceanic bacterium. Science **309**:1242-1245.

Glaeser, S. P. and P. Kämpfer. 2014. The Family Sphingomonadaceae. Pages 641-707 The Prokaryotes. Springer.

Grzymski, J. J. and A. M. Dussaq. 2012. The significance of nitrogen cost minimization in proteomes of marine microorganisms. The ISME journal **6**:71-80.

Güde, H. 1979. Grazing by protozoa as selection factor for activated sludge bacteria. Microbial Ecology **5**:225-237.

Hahn, M., E. Moore, and M. Höfle. 2000. Role of microcolony formation in the protistan grazing defense of the aquatic bacterium Pseudomonas sp. MWH1. Microbial Ecology **39**:175-185.

Hahn, M. W. and M. G. Höfle. 1999. Flagellate Predation on a Bacterial Model Community: Interplay of Size-Selective Grazing, Specific Bacterial Cell Size, and Bacterial Community Composition. Applied and Environmental Microbiology **65**:4863-4872.

Hahn, M. W. and M. G. Höfle. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiology Ecology **35**:113-121.

Halligan, D. L. and P. D. Keightley. 2009. Spontaneous mutation accumulation studies in evolutionary genetics. Annual Review of Ecology, Evolution, and Systematics **40**:151-172.

Hillenmeyer, M. E., E. Fung, J. Wildenhain, S. E. Pierce, S. Hoon, W. Lee, M. Proctor, R. P. S. Onge, M. Tyers, and D. Koller. 2008. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science **320**:362-365.

Houle, D., D. Hoffmaster, S. Assimacopoulos, and B. Charlesworth. 1994. The genomic mutation rate for fitness in Drosophila. Nature **371**:358.

Janssen, P. H., A. Schuhmann, E. Mörschel, and F. A. Rainey. 1997. Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. Applied and Environmental Microbiology **63**:1382-1388.

Jeschke, J. M., M. Kopp, and R. Tollrian. 2002. Predator functional responses: discriminating between handling and digesting prey. Ecological Monographs **72**:95-112.

Jogler, M., H. Siemens, H. Chen, B. Bunk, J. Sikorski, and J. Overmann. 2011. Identification and Targeted Cultivation of Abundant Novel Freshwater Sphingomonads and Analysis of Their Population Substructure. Applied and Environmental Microbiology **77**:7355-7364.

Jousset, A. 2012. Ecological and evolutive implications of bacterial defences against predators. Environmental Microbiology **14**:1830-1843.

Jousset, A., E. Lara, L. G. Wall, and C. Valverde. 2006. Secondary metabolites help biocontrol strain Pseudomonas fluorescens CHA0 to escape protozoan grazing. Applied and Environmental Microbiology **72**:7083-7090.

Jousset, A., L. Rochat, S. Scheu, M. Bonkowski, and C. Keel. 2010. Predator-prey chemical warfare determines the expression of biocontrol genes by rhizosphere-associated Pseudomonas fluorescens. Applied and Environmental Microbiology **76**:5263-5268.

Jürgens, K. and H. Güde. 1994. The potential importance of grazing-resistant bacteria in planktonic systems. Marine Ecology Progess Series **112**:169-188.

Jürgens, K. and E. Jeppesen. 2000. The impact of metazooplankton on the structure of the microbial food web in a shallow, hypertrophic lake. Journal of Plankton Research **22**:1047-1070.

Jürgens, K., J. Pernthaler, S. Schalla, and R. Amann. 1999. Morphological and Compositional Changes in a Planktonic Bacterial Community in Response to Enhanced Protozoan Grazing. Applied and Environmental Microbiology **65**:1241-1250.

Jürgens, K. and K. Šimek. 2000. Functional response and particle size selection of Halteria cf. grandinella, a common freshwater oligotrichous ciliate. Aquatic Microbial Ecology **22**:57-68.

Justice, S. S., D. A. Hunstad, L. Cegelski, and S. J. Hultgren. 2008. Morphological plasticity as a bacterial survival strategy. Nature Reviews Microbiology **6**:162-168.

Kawahara, K., U. Seydel, M. Matsuura, H. Danbara, E. T. Rietschel, and U. Za. 1991. Chemical structure of glycosphingolipids isolated fromSphingomonas paucimobilis. FEBS letters **292**:107-110.

Kawecki, T. J., R. E. Lenski, D. Ebert, B. Hollis, I. Olivieri, and M. C. Whitlock. 2012. Experimental evolution. Trends in Ecology & Evolution **27**:547-560.

Klasson, L. and S. G. E. Andersson. 2004. Evolution of minimal-gene-sets in host-dependent bacteria. Trends in Microbiology **12**:37-43.

Kolss, M., R. K. Vijendravarma, G. Schwaller, and T. J. Kawecki. 2009. LIFE-HISTORY CONSEQUENCES OF ADAPTATION TO LARVAL NUTRITIONAL STRESS IN DROSOPHILA. Evolution **63**:2389-2401.

Koonin, E. V., K. S. Makarova, and L. Aravind. 2001. Horizontal Gene Transfer in Prokaryotes: Quantification and Classification. Annual Review of Microbiology **55**:709-742.

Koonin, E. V. and Y. I. Wolf. 2008. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. Nucleic Acids Research **36**:6688-6719.

Kosako, Y., E. Yabuuchi, T. Naka, N. Fujiwara, and K. Kobayashi. 2000. Proposal of Sphingomonadaceae fam. nov., consisting of Sphingomonas Yabuuchi et al. 1990, Erythrobacter Shiba and Shimidu 1982, Erythromicrobium Yurkov et al. 1994, Porphyrobacter Fuerst et al. 1993, Zymomonas Kluyver and van Niel 1936, and Sandaracinobacter Yurkov et al. 1997, with the type genus Sphingomonas Yabuuchi et al. 1990. Microbiology and immunology **44**:563-575.

Labrie, S. J., J. E. Samson, and S. Moineau. 2010. Bacteriophage resistance mechanisms. Nat Rev Micro **8**:317-327.

Laforsch, C. and R. Tollrian. 2004. Extreme helmet formation in Daphnia cucullata induced by small-scale turbulence. Journal of Plankton Research **26**:81-87.

Lee, C. E., J. L. Remfert, and Y.-M. Chang. 2007. Response to selection and evolvability of invasive populations. Genetica **129**:179-192.

Lee, M.-C. and C. J. Marx. 2012. Repeated, Selection-Driven Genome Reduction of Accessory Genes in Experimental Populations. PLoS Genet **8**:e1002651.

Lenski, R. E. 1988. Experimental studies of pleiotropy and epistasis in Escherichia coli. I. Variation in competitive fitness among mutants resistant to virus T4. Evolution:425-432.

Lenski, R. E. and B. R. Levin. 1985. Constraints on the Coevolution of Bacteria and Virulent Phage: A Model, Some Experiments, and Predictions for Natural Communities. The American Naturalist **125**:585-602.

Lenski, R. E., C. L. Winkworth, and M. A. Riley. 2003. Rates of DNA Sequence Evolution in Experimental Populations of Escherichia coli During 20,000 Generations. Journal of Molecular Evolution **56**:498-508.

Lewis, I. M. 1934. Bacterial variation with special reference to behavior of some mutabile strains of colon bacteria in synthetic media. Journal of Bacteriology **28**:619-639.

Liu, X., Z. Gai, F. Tao, H. Tang, and P. Xu. 2012. Carotenoids Play a Positive Role in the Degradation of Heterocycles by <italic>Sphingobium yanoikuyae</italic>. PLoS ONE **7**:e39522.

Liu, Y., Q. Hou, W. Liu, Y. Meng, and G. Wang. 2015. Dynamic changes of bacterial community under bioremediation with Sphingobium sp. LY-6 in buprofezin-contaminated soil. Bioprocess and biosystems engineering:1-9.

Lubin, Y., J. R. Henschel, and M. B. Baker. 2001. Costs of aggregation: shadow competition in a sit-and-wait predator. Oikos **95**:59-68.

Luria, S. E. and M. Delbrück. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491.

Matz, C., J. Boenigk, H. Arndt, and K. Jürgens. 2002. Role of bacterial phenotypic traits in selective feeding of the heterotrophic nanoflagellate Spumella sp. Aquatic Microbial Ecology **27**:137-148.

Matz, C. and S. Kjelleberg. 2005. Off the hook–how bacteria survive protozoan grazing. Trends in Microbiology **13**:302-307.

Matz, C., D. McDougald, A. M. Moreno, P. Y. Yung, F. H. Yildiz, and S. Kjelleberg. 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **102**:16819-16824.

Maughan, H. and W. L. Nicholson. 2011. Increased Fitness and Alteration of Metabolic Pathways during Bacillus subtilis Evolution in the Laboratory. Applied and Environmental Microbiology **77**:4105-4118.

McAdams, H. H., B. Srinivasan, and A. P. Arkin. 2004. The evolution of genetic regulatory systems in bacteria. Nature Reviews Genetics **5**:169-178.

Miller, K., A. J. O'Neill, and I. Chopra. 2002. Response of Escherichia coli hypermutators to selection pressure with antimicrobial agents from different classes. Journal of Antimicrobial Chemotherapy **49**:925-934.

Miller, M. B. and B. L. Bassler. 2001. Quorum sensing in bacteria. Annual Reviews in Microbiology **55**:165-199.

Mira, A., H. Ochman, and N. A. Moran. 2001. Deletional bias and the evolution of bacterial genomes. Trends in Genetics **17**:589-596.

Moran, N. A. 2002. Microbial Minimalism: Genome Reduction in Bacterial Pathogens. Cell **108**:583-586.

Morgan, T. H. 1910. Sex Limited Inheritance in Drosophila. Science **32**:120-122.

Morris, S. C. 1998. Early metazoan evolution: reconciling paleontology and molecular biology. American Zoologist **38**:867-877.

Muyzer, G. and N. B. Ramsing. 1995. Molecular methods to study the organization of microbial communities. Water Science and Technology **32**:1-9.

Nakayama, K., K. Takashima, H. Ishihara, T. Shinomiya, M. Kageyama, S. Kanaya, M. Ohnishi, T. Murata, H. Mori, and T. Hayashi. 2000. The R-type pyocin of Pseudomonas aeruginosa is related to P2 phage, and the F-type is related to lambda phage. Molecular Microbiology **38**:213-231.

Nilsson, A. I., S. Koskiniemi, S. Eriksson, E. Kugelberg, J. C. D. Hinton, and D. I. Andersson. 2005. Bacterial genome size reduction by experimental evolution. Proceedings of the National Academy of Sciences of the United States of America **102**:12112-12116.

Ochman, H. 2005. Genomes on the shrink. Proceedings of the National Academy of Sciences of the United States of America **102**:11959-11960.

Ochman, H. and L. M. Davalos. 2006. The Nature and Dynamics of Bacterial Genomes. Science **311**:1730-1733.

Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405**:299-304.

Ohno, S. 1972. So much "junk" DNA in our genome. Pages 366-370 *in* Brookhaven Symp Biol.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. Science **276**:734-740.
Pal, C., M. D. Maciá, A. Oliver, I. Schachar, and A. Buckling. 2007. Coevolution with viruses drives the evolution of bacterial mutation rates. Nature **450**:1079-1081.
Palumbi, S. R. 2001. Humans as the World's Greatest Evolutionary Force. Science **293**:1786-1790.
Partensky, F., W. R. Hess, and D. Vaulot. 1999. Prochlorococcus, a Marine Photosynthetic Prokaryote of Global Significance. Microbiology and Molecular Biology Reviews **63**:106-127.
Patterson, C. 1981. Significance of fossils in determining evolutionary relationships. Annual Review Of Ecology And Systematics:195-223.
Pernthaler, J. 2005. Predation on prokaryotes in the water column and its ecological implications. Nature Reviews Microbiology **3**:537-546.
Pernthaler, J., B. Sattler, K. Simek, A. Schwarzenbacher, and R. Psenner. 1996. Top-down effects on the size-biomass distribution of a freshwater bacterioplankton community. Aquatic Microbial Ecology **10**:255-263.
Pernthaler, J., E. Zollner, F. Warnecke, and K. Jurgens. 2004. Bloom of filamentous bacteria in a mesotrophic lake: Identity and potential controlling mechanism. Applied and Environmental Microbiology **70**:6272-6281.
Perron, G. G., M. Zasloff, and G. Bell. 2006. Experimental evolution of resistance to an antimicrobial peptide. Proceedings of the Royal Society B: Biological Sciences **273**:251-256.
Posch, T., K. Simek, J. Vrba, J. Pernthaler, J. Nedoma, B. Sattler, B. Sonntag, and R. Psenner. 1999. Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. Aquatic Microbial Ecology **18**:235-246.
Postma, J., C. H. Hok-A-Hin, and J. A. van Veen. 1990. Role of Microniches in Protecting Introduced Rhizobium leguminosarum biovar trifolii against Competition and Predation in Soil. Applied and Environmental Microbiology **56**:495-502.
Pradel, E., Y. Zhang, N. Pujol, T. Matsuyama, C. I. Bargmann, and J. J. Ewbank. 2007. Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans. Proceedings of the National Academy of Sciences **104**:2295-2300.
Rainey, P. B. and M. Travisano. 1998. Adaptive radiation in a heterogeneous environment. Nature **394**:69-72.
Rambow-Larsen, A. A., G. Rajashekara, E. Petersen, and G. Splitter. 2008. Putative Quorum-Sensing Regulator BlxR of Brucella melitensis Regulates Virulence Factors Including the Type IV Secretion System and Flagella. Journal of Bacteriology **190**:3274-3282.
Ranea, J. A. G., A. Grant, J. M. Thornton, and C. A. Orengo. 2005. Microeconomic principles explain an optimal genome size in bacteria. Trends in Genetics **21**:21-25.
Rogers, D. W. and D. Greig. 2009. Experimental evolution of a sexually selected display in yeast. Proceedings of the Royal Society of London B: Biological Sciences **276**:543-549.
Rønn, R., A. E. McCaig, B. S. Griffiths, and J. I. Prosser. 2002. Impact of protozoan grazing on bacterial community structure in soil microcosms. Applied and Environmental Microbiology **68**:6094-6105.
Salcher, M. M., S. M. Neuenschwander, T. Posch, and J. Pernthaler. 2015. The ecology of pelagic freshwater methylotrophs assessed by a high-resolution monitoring and isolation campaign. The ISME journal.
Salcher, M. M., J. Pernthaler, and T. Posch. 2011. Seasonal bloom dynamics and ecophysiology of the freshwater sister clade of SAR11 bacteria 'that rule the waves' (LD12). Isme Journal **5**:1242-1252.
Shilo, M. and B. Bruff. 1965. LYSIS OF GRAM-NEGATIVE BACTERIA BY HOST-INDEPENDENT ECTOPARASITIC BDELLOVIBRIO BACTERIOVORUS ISOLATES. Journal of General Microbiology **40**:317-&.
Simek, K., P. Kojecka, J. Nedoma, P. Hartman, J. Vrba, and J. R. Dolan. 1999. Shifts in bacterial community composition associated with different microzooplankton size fractions in a eutrophic reservoir. Limnology and Oceanography **44**:1634-1644.

Smith, S. V. 1984. Phosphorus versus nitrogen limitation in the marine environment1. Limnology and Oceanography **29**:1149-1160.

Sniegowski, P. D., P. J. Gerrish, and R. E. Lenski. 1997. Evolution of high mutation rates in experimental populations of E. coli. Nature **387**:703-705.

Sowell, S. M., L. J. Wilhelm, A. D. Norbeck, M. S. Lipton, C. D. Nicora, D. F. Barofsky, C. A. Carlson, R. D. Smith, and S. J. Giovanonni. 2008. Transport functions dominate the SAR11 metaproteome at low-nutrient extremes in the Sargasso Sea. ISME J **3**:93-105.

Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace. 1985. CHARACTERIZATION OF A YELLOWSTONE HOT-SPRING MICROBIAL COMMUNITY BY 5S RIBOSOMAL-RNA SEQUENCES. Applied and Environmental Microbiology **49**:1379-1384.

Sutherland, I. W. 2001. Microbial polysaccharides from Gram-negative bacteria. International Dairy Journal **11**:663-674.

Tansley, A. G. and R. S. Adamson. 1925. Studies of the vegetation of the English chalk: III. The chalk grasslands of Hampshire-Sussex border. The Journal of Ecology:177-223.

Tecon, R. and J. R. van der Meer. 2010. Effect of two types of biosurfactants on phenanthrene availability to the bacterial bioreporter Burkholderia sartisoli strain RP037. Applied microbiology and biotechnology **85**:1131-1139.

Thingstad, T. F. 2000. Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. Limnology and Oceanography **45**:1320-1328.

Thingstad, T. F., L. Øvreås, J. K. Egge, T. Løvdal, and M. Heldal. 2005. Use of non-limiting substrates to increase size; a generic strategy to simultaneously optimize uptake and minimize predation in pelagic osmotrophs? Ecology Letters **8**:675-682.

Thomason, L. C., J. A. Sawitzke, X. Li, N. Costantino, and D. L. Court. 2001. Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination. Current Protocols in Molecular Biology. John Wiley & Sons, Inc.

Tiirola, M. A., M. K. Männistö, J. A. Puhakka, and M. S. Kulomaa. 2002. Isolation and Characterization of Novosphingobium sp. Strain MT1, a Dominant Polychlorophenol-Degrading Strain in a Groundwater Bioremediation System. Applied and Environmental Microbiology **68**:173-180.

van der Woude, M. W. and A. J. Baumler. 2004. Phase and antigenic variation in bacteria. Clinical Microbiology Reviews **17**:581-+.

Vaqué, D. and M. L. Pace. 1992. Grazing on bacteria by flagellates and cladocerans in lakes of contrasting food-web structure. Journal of Plankton Research **14**:307-321.

Vaz-Moreira, I., O. C. Nunes, and C. M. Manaia. 2011. Diversity and Antibiotic Resistance Patterns of Sphingomonadaceae Isolates from Drinking Water. Applied and Environmental Microbiology **77**:5697-5706.

Vorholt, J. A. 2012. Microbial life in the phyllosphere. Nat Rev Micro **10**:828-840.

Vos, M. 2009. Why do bacteria engage in homologous recombination? Trends in Microbiology **17**:226-232.

WHO. 2000. Benzoic acid and sodium benzoate, World Health Organization, Concise International Chemical Assessment Document 26, http://www.who.int/ipcs/publications/cicad/cicad26_rev_1.pdf.

Wilhelm, S. W. and C. A. Suttle. 1999. Viruses and Nutrient Cycles in the Sea: Viruses play critical roles in the structure and function of aquatic food webs. BioScience **49**:781-788.

Willison, J. C. 2004. Isolation and characterization of a novel sphingomonad capable of growth with chrysene as sole carbon and energy source. FEMS Microbiology Letters **241**:143-150.

Wommack, K. E. and R. R. Colwell. 2000. Virioplankton: Viruses in aquatic ecosystems. Microbiology and Molecular Biology Reviews **64**:69-+.

Yabuuchi, E. and Y. Kosako. 2015. Sphingomonadaceae. Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Ltd.

Yamamichi, M., T. Yoshida, and A. Sasaki. 2011. Comparing the effects of rapid evolution and phenotypic plasticity on predator-prey dynamics. The American Naturalist **178**:287-304.

7 Curriculum Vitae

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Education

2012-2016	**University of Zurich**, Institute of plant and microbiology PhD in Evolutionary biology (Funded by the University research priority program "Evolution in action") Thesis Title: "Experimental evolution of a freshwater isolate with inducible aggregate formation – Genome streamlining and variability of phenotypic plasticity." Supervisor: Professor Jakob Pernthaler and Dr. Judith Blom
2010-2012	**University of Zurich**, Institute of plant and microbiology Master of Science in Biology Thesis title: "Aggregate formation and evolutionary adaptation to predation in *Sphingobium* sp. isolates"
2007-2010	**University of Zurich**, Institute of plant and microbiology Bachelor of Science in Biology
2002-2006	Kantonsschule Enge, Zürich Matura (specialisation in Economics & Law),

Publications

2016	Michael Baumgartner, Jakob Pernthaler (2016) *Adaptive phenotypic defence is only preserved in a bacterial strain at alternating conditions of predator presence and absence.* (Submitted)
2016	Michael Baumgartner, Stefan Roffler, Thomas Wicker, Jakob Pernthaler (2016) *Letting go: genome streamlining allows for simultaneous adaptation to top-down and bottom-up constraints in a phenotypically plastic bacterial strain.* (Submitted)

2016	Michael Baumgartner, Thomas R. Neu, Judith F. Blom, Jakob Pernthaler (2016) *Predation on a bacterial strain outweighs substrate limitation in selecting for defence over growth optimized morphotypes* (Submitted)
2013	Ester M. Eckert, Michael Baumgartner, Iris M. Huber and Jakob Pernthaler (2013) *Grazing resistant freshwater bacteria profit from chitin and cell-wall-derived organic carbon*. Environmental Microbiology

Teaching

2011-2015	Practical course in Aquatic Microbial Ecology, UZH
2007-present	Tutor in mathematics, High school level

Additional relevant experiences

2013	**University of Greifswald**, International proteomics summer school
2011	**Helmholtz Centre for Environmental Research**, Magdeburg, Training in confocal laser microscopy and lectin staining, supervised by Dr. T. Neu; Department of River Ecology
2010	**Oceans Research Marine Lab**, Mossel Bay, South Africa, Internship in ecology and conservational biology

Awards

2015	Best student oral presentation, Conference of the society for aquatic microbial ecology, Uppsala Sweden

Presentations and Posters

2015	Baumgartner M et al: "*Genome streamlining in bacteria evolving under high predation pressure*". Oral presentation at the Conference of the society for aquatic microbial ecology, Uppsala Sweden
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2014	Baumgartner M et al: "*Low substrate concentration and predator-prey interactions induce rapid adaptations in a freshwater bacterial isolate*": Poster presentation at the conference of the international society of microbial ecology, Seoul, South Korea
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